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Title: Induction of Broad Immunity by Thermostabilised Vaccines Incorporated in Dissolvable Microneedles Using Novel Fabrication Methods

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Keywords: Microneedle, influenza vaccine, broadly-neutralizing antibody, stability, virus vector vaccine

Corresponding Author: Dr. Anne C Moore, Ph.D.

Corresponding Author's Institution: University College Cork

First Author: Anto Vrdoljak, PhD

Order of Authors: Anto Vrdoljak, PhD; Evin Allen; Francesca Ferrara; Nigel J Temperton; Abina M Crean ; Anne C Moore, Ph.D.

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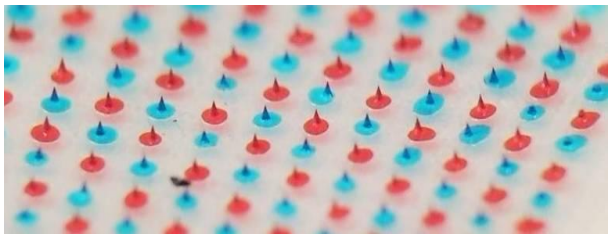
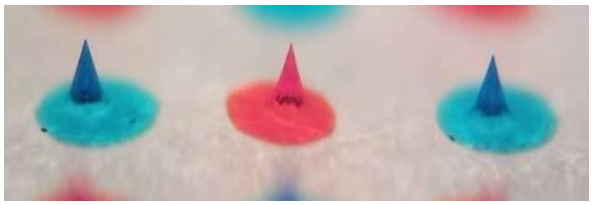
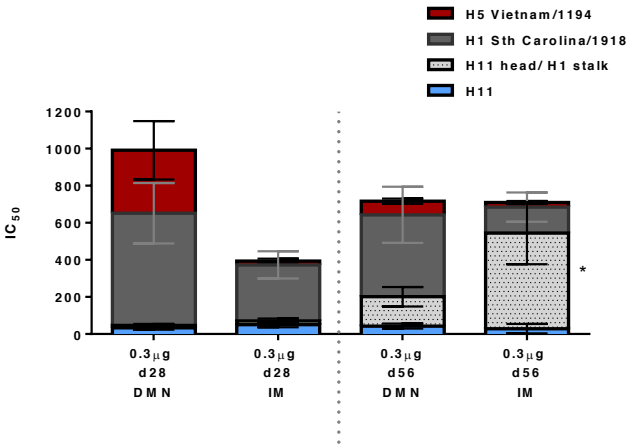
Graphical Abstract

Precisely dispensing formulation onto microneedle moulds permits the production of heterogeneous patches without wasted material. Influenza vaccine-loaded microneedle patches induce broader neutralising antibody responses compared to intramuscular injection.







Induction of Broad Immunity by Thermostabilised Vaccines Incorporated in Dissolvable Microneedles Using Novel Fabrication Methods

Short title: Immunity of Stabilized Vaccine in Microneedles

Anto Vrdoljak[a], Evin A. Allen[a], Francesca Ferrara[b], Nigel J. Temperton[b], Abina M. Crean[a] Anne C. Moore[a, c, *]

[a]School of Pharmacy, University College Cork, Cork, Ireland.

[b]School of Pharmacy, University of Kent, the Medway, UK.

[c]Department of Pharmacology, University College Cork, Cork, Ireland.

*Corresponding Author: Anne Moore.

School of Pharmacy, Cavanagh Pharmacy Building, University College Cork, Cork, Ireland.

T: +353-21-4901665

E: anne.moore@ucc.ie

Abstract

Dissolvable microneedle (DMN) patches for immunization have multiple benefits, including vaccine stability and ease-of-use. However, conventional DMN fabrication methods have several drawbacks. Here we describe a novel, microfluidic, drop dispensing-based dissolvable microneedle production method that overcomes these issues. Uniquely, heterogeneous arrays, consisting of microneedles of diverse composition can be easily produced on the same patch. Robustness of the process was demonstrated by incorporating and stabilizing adenovirus and MVA vaccines. Clinically-available trivalent inactivated influenza vaccine (TIV) in DMN patches is fully stable for greater than 6 months at 40ºC. Immunization using low dose TIV-loaded DMN patches induced significantly higher antibody responses compared to intramuscular-based immunization in mice. TIV-loaded patches also induced a broader, heterosubtypic neutralising antibody response. By addressing issues that will be faced in large-scale fill-finish DMN fabrication processes and demonstrating superior thermostable characteristics and immunogenicity, this study progresses the translation of this microneedle platform to eventual clinical deployment.

Keywords: Microneedle, influenza vaccine, broadly-neutralizing antibody, stability, virus vector vaccine

Introduction

Although routinely used, systemic vaccine injection has several drawbacks. Hypodermic needle injection is painful, requires trained personnel, generates hazardous sharps-waste and the vaccine requires cold-chain storage and distribution [1-3]. This results in an unsustainable financial and logistic costs to many immunization programs [1, 2]. Vaccine-loaded dissolvable microneedles are micron-scale protrusions that are sharp enough to insert into the skin. They

are composed of suitable excipients that should stabilise vaccines in the dry, solid state. On skin insertion the microneedle material dissolves and the vaccine is delivered to the body. Dissolvable microneedle (DMN) patches for skin-based immunization offer several benefits to healthcare systems and individual end-users including ease of use, lack of requirement for vaccine reconstitution, more efficient and cost-effective logistics by removal of cold chain, elimination of needles, syringes and hazardous waste and small package size.

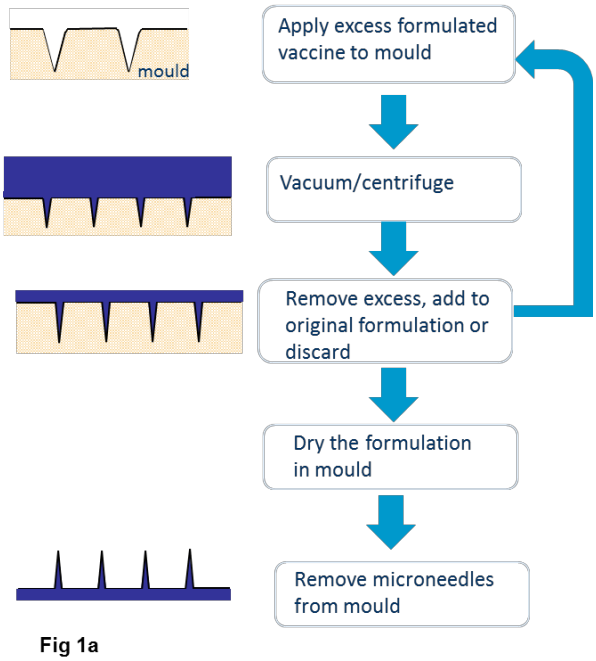


Fig 1a

Several DMN patch production methods have been proposed [4-8]. Most of these methods rely on filling a liquid formulation into a mould, having microdepressions or pores that define the final structure and composition of the microneedles, and subsequent drying and/or hardening of the material **(Fig. 1a**). Filling the liquid formulation into the microdepressions is not spontaneous due to the sub-micron dimensions of the tips, surface tension and often high viscosity of the liquid formulation. Incomplete filling of the microneedle mould results in poor microneedle tip formation or an inability to remove the dissolvable microneedle from the mould (**Fig. 1d**). Approaches that are most often used to overcome this problem include processes such as centrifuging, pressurizing or vacuuming of the mould with the formulation deposited on its top and/or inclusion of surfactants in the formulation [9-11]. When dry, the solid material, which has taken the shape of the mould, is pulled out (**Fig 1a**). One of the principle drawbacks of these methods is the need to apply larger volumes of formulation onto the microneedle moulds in the filling step where only a fraction of the volume used actually fills the microneedle pores while the rest remains unused. Although the formulation remaining on

the surface of the moulds may be reused, such recycling approaches may imply compliance issues in a Good Manufacturing Practice (cGMP) environment. Therefore, although a proposed advantage of DMN patches is dose sparing at the point of use, current methods of fabrication result in wasted vaccine at the point of manufacture. Other drawbacks of this technology at an industrial scale include the reliance on batch-based/semi-continuous process operations (centrifuging, pressurizing or vacuuming) inhibiting its scale-up to a flexible, continuous manufacturing process and the difficulty of easily creating heterogeneous patches containing individual microneedles that are composed of different materials. Here we describe a novel process that avoids waste, is designed for scalability using existing automated micro-dispensing systems, and is amenable for continuous manufacture. We believe that designing a fabrication process with these advantages increases the potential for translation from preclinical scale to commercial scale manufacture for the manufacturer stakeholder. We solved the problem of wasted vaccine by only dispensing the formulation on top of the microneedle cavities [12]. Issues of incomplete filling of the microneedle cavities, due to surface tension of the formulation with the mould are overcome by pre-filling the mould with water and dispensing the water-based, vaccine-containing formulation onto each microdepression. The resulting microneedles completely replicate the structure of the original master template; the DMN had smooth walls and sharp tips. As the microdepressions on these microneedle patches are filled in a sequential mode this opens the possibility for making heterogeneous patches consisting of microneedles made of different formulations. Similar to other DMN production methods, the described method can also easily produce DMN with formulation concentrated in the microneedle tips.

Having defined this novel process, the next objective was to determine the potential utility of DMN patches incorporating clinically relevant vaccines. We characterized the thermostability of labile recombinant live virus vaccines that are dependent on retention of sensitive bio-physicochemical properties for immunogenicity [13]. These vaccines are in general less stable

than inactivated vaccines. Subsequently, we determined the thermostability and immunogenicity of the clinically licensed 2011/2012 seasonal trivalent inactivated influenza vaccine (TIV) when incorporated in DMN patches. This study therefore demonstrates that an efficient process for manufacturing dissolvable microneedle patches has strong potential for stabilizing clinically relevant vaccines outside of cold chain and for retaining and broadening vaccine immunogenicity after incorporation into dissolvable microneedles.

MATERIALS AND METHODS

Manufacture of PDMS microneedle moulds

Silicon microneedle master templates were prepared by wet-etch process using a previously reported approach [14]. For this study pyramidal silicon microneedles arranged on arrays of 12 x 12 (length: 280 μm, base diameter: 187 μm) or 5 x 5 (length: 500 μm, base diameter: 333 μm) microneedles were used as a moulding templates for fabrication of microneedle cavities in polydimethylsiloxane (PDMS) moulds. The PDMS moulds were prepared as previously described [15].

Vaccines and formulations

Trehalose, methylene blue, Congo red were from Sigma; polyvinylalcohol (PVA, molecular weight 130000) from Kurray and polyvinylpyrrolidone (PVP) was supplied from BASF. MVA encoding the red fluorescent protein (MVA-RFP) or β-galactosidase (MVA-β-gal) and adenovirus serotype 5 encoding mCherry protein (AdV-mCherry) were kindly provided by The Jenner Institute, Oxford, UK. AdV expressing β-galactosidase was procured from Clontech. Influvac 2011-12 Northern Hemisphere (NH) (Abbott) and 2011-12 Fluarix NH (GSK) vaccines were used in these studies. All vaccines contained 15μg of haemagglutinin from each of the following strains: A/California/7/2009 (H1N1)-derived strain using NYMC X-181, A/Perth/16/2009 (H3N2)-like strain used NYMC X-187 derived from A/Victoria/210/2009 and B/Brisbane/60/2008. The

vaccine was concentrated, using Amicon centrifugal filter units (Millipore) with a 10kDa specification, when a dose of 1.5 µg of each HA was incorporated into a 1cm^2 patch. It was not concentrated when the lower dosage of 0.375 µg of each HA was used. The vaccine was concentrated 7.9 times prior to formulation, to create a vaccine with a HA concentration of 238 µg/ml (compared to initial 30 µg/ml). Formulation was then added to this concentrated vaccine so that 1.5 µg of each HA was loaded into each 1cm^2 microneedle mould.

Dissolvable microneedle fabrication

For the preparation of microneedles with a single formulation throughout, either 50% (w/v) trehalose solution in water or a combination of 25% trehalose and 7.5% polyvinyl alcohol (w/v) in water was used. For the preparation of arrays with two different formulations 25% trehalose (w/v) in water solution was used for making the microneedle tips and 40% (w/v) (PVP) in 96% ethanol (v/v) was used in the base. Methylene blue, Congo red dye (1 mg/mL) or red fluorescent latex microspheres with 0.1 µm diameter (F-8801, Invitrogen) were added for visualization experiments, where appropriate. Microneedle cavities were filled with water by spraying using two substance nozzle and compressed air (Düsen-Schlick 970 S8, Germany). Excess water on the surface of the mould was scrapped off the mould using a blade. Formulation was delivered directly onto the water-filled microneedle pores using a thin silicon capillary (100 µm ID) connected to a syringe pump delivering formulation at a rate of 1-3 µL/min. Following delivery of the formulation onto moulds, microneedles were dried for 2 hours or overnight at room temperature (approximately 21ºC) in the presence of dessicant. After transfer onto medical grade adhesive tape (1525L Poly Med tape, 3M) arrays were kept in a dry ambient environment in the presence of dessicant. Two-layered microneedles, where the active material is concentrated to the tip, were formed by partially filling the pore. Partial filling of the microneedle tip was achieved by either using the same volume of less concentrated formulation containing a lower amount of solutes or by using a smaller volume of more concentrated solution. Both

options delivered an amount of final dry matter sufficient for only partial filling of the tip resulting in microneedles with the formulation concentrated in the tip region only. After drying a second, inert layer of, for example, polyvinylpyrrolidone (PVP), can be applied to the mould to fill the remaining microneedle pore volume.

Microneedle patches were visualized either using light microscope or a 10x magnifier attached to a photo camera. Patches containing fluorescent microspheres were photographed using fluorescent microscope (Nikon). Visualization of the drying process was performed on PDMS moulds with 280 μm tall microneedle pores. Formulation consisting of 45% (w/v) trehalose and methylene blue dye was delivered on PDMS mould and immediately positioned under the microscope. The drying process was recorded for 20 min taking photographs in regular intervals.

Kinetics of dissolution of dissolvable microneedles

Kinetics of dissolution of microneedles was performed using cadaver pig skin. Arrays of 500 μm tall microneedles were prepared as described with microneedle tips made of trehalose with Congo red dye and microneedles bases made of PVP with methylene blue. Following drying, patches were applied onto previously shaved pig skin and left for 1 s, 10 min or 60 min in the skin kept at 37 °C. Patches and skin were imaged using a light microscope after patch removal from the skin.

Skin-transfection studies

AdV and MVA expressing β-galactosidase were embedded in the microneedles at the approximate concentration of $1.5x10^4$ pfu per microneedle. Freshly excised pig skin was used for the *ex vivo* transfection studies essentially as described [13] .

Vaccine Stability Studies

MVA-RFP was formulated in 25% trehalose with 7.5% PVA (w/v) solution at a starting concentration of 10^9 pfu/mL. AdV-mCherry was formulated in 50% (w/v) trehalose solution at the

concentration of 2 x 10^9 pfu/mL. FITC-Na was added in all virus vaccine formulations at the concentration of 1 mg/mL to enable precise quantification of the amount of formulation delivered onto each individual mould. TIV-loaded DMN patches were fabricated using 11% trehalose (w/v) and 2.75% PVA (w/v), and 1.5 µg of each of the three HA antigens, unless otherwise stated. Patches containing test components were sealed into nitrogen-purged individual glass vials with dessicant. Packaged and sealed vaccine-loaded DMN were stored in a stability chamber at 40°C, with the surrounding chamber maintained at 75% relative humidity (RH), in accordance with ICH guidelines, for the indicated time.

Assessing Live Vaccine Viability

Virus vaccines were kept at ambient temperature for up to 14 days. Survival of AdV and MVA expressing fluorescent proteins was measured using flow cytometry. Arrays of microneedles were dissolved in cell culture medium at ambient temperature. DF-1 (MVA-RFP) or HEK293A (AdV-mCherry) cells grown under standard conditions were infected with virus solutions and left overnight in CO_2 incubator. After 24 hours cells were harvested and infection rate was calculated by measuring fluorescence of infected cells expressing RFP or mCherry proteins using LSRII flow cytometer (Becton-Dickinson). Survival rate was calculated from standard curve using samples of known titer (in PFU/mL units) and was expressed as log PFU_{eq}/mL units (logarithmic value of plaque forming unit equivalents per mL) [13].

Single Radial Immunodiffusion (SRID)

This was performed as previously reported [16, 17]. Briefly detergent pre-treatment of samples and reference agents was performed with a 10% (w/v) Zwittergent 3-14 detergent solution (Sigma) to a final concentration of 1% detergent. Serially diluted sample mixtures were loaded into appropriate wells of a 1% (w/w) agarose gel, containing vaccine strain specific HA antiserum (National Institute for Biological Standards and Control) and incubated for 18 hours at RT. The gel was stained using coomassie brilliant blue (Invitrogen) according to manufacturer's

instructions followed by destaining with deionised water. Immunodiffusion rings were then measured and analysed using the slope-ratio method [18] to determine the HA content, according to regulatory approved bioassay analysis method.

Immunisation Studies

Hair was removed from the ears of mice using depilatory cream two days before immunization. On the day of immunization a 1cm^2 patch was applied to each ear of female 6–8 week old BALB/c mice and pressed, by hand, using a force of approximately 10–20 N per patch, in a vertical direction [15, 19, 20]. Patches were macroscopically inspected after removal from the ear and no material was found on them; this provided qualitative assurance that microneedles had dissolved from the adhesive backing. The efficiency of vaccine delivery into skin was not quantified. Patches were maintained in place using an adhesive fabric strip for 18 hours before being removed. Alternatively, liquid vaccine was injected by the intramuscular route (25 μL into each gastrocnemius muscle). All murine experiments were conducted in strict accordance with the terms of licences from the Irish Department of Health and Children, under the Cruelty to Animals Act 1876 (licence numbers B100/4034 and B100/4478) and according to the approval of the UCC AECC committee.

Antibody ELISA

Serum antigen-specific IgG responses were assessed as previously described [20]. The following reagents were obtained through BEI Resources, NIAID, NIH: H1 Hemagglutinin (HA) protein from Influenza Virus, A/California/04/2009 (H1N1)pdm09, recombinant from Baculovirus, NR-13691 and Hemagglutinin (HA) protein with C-Terminal histidine tag from Influenza Virus, A/Perth/16/2009 (H3N2), recombinant from Baculovirus, NR-42974. Briefly, Nunc C maxisorp plates were coated with trivalent influenza vaccine (TIV) at a concentration of 0.75 μg/ml or recombinant A/California/7/2009 (H1N1) or A/Perth/16/2009 (H3N2) at a concentration of 1 μg/ml in carbonate bicarbonate buffer overnight at 4°C. After blocking, sera were serially diluted

on the plate. After 2 hours incubation, anti-mouse IgG-HRP antibody at a dilution of 1:10,000 was added for one hour. Plates were washed and incubated with 50µl of TMB for thirty minutes. Plates were then read at 655nm and titres were determined using endpoint titre method [20].

Determination of neutralising antibodies using lentiviral pseudotypes

Pseudotype assays were performed as previously described [21-23]. Briefly, influenza lentiviruses expressing firefly luciferase and harbouring A/South Carolina/1/1918 (H1N1) A/Udorn/307/1972 (H3N2) and A/Vietnam/1203/2004 (H5N1) HAs were used initially. Then to assess the presence of stalk-directed antibodies lentiviruses bearing the A/duck/Memphis/546/1974 (H11N9) HA or a chimeric HA consisting of the H11 head and the A/South Carolina/1/1918 H1 stalk were used. Mouse serum samples were serially diluted twofold from 1:40 in a 96-well flat bottom plate. A comparable amount of each pseudotype (giving an output of 1,000,000 Relative Light Units) was incubated with sera for 1 h at 37°C before the addition of 1.5×10^4 293T/17 cells per well. Luciferase activity was measured 48 h later and IC_{50} neutralization titre was determined using GraphPad Prism as the serum dilution yielding a 50% reduction in luciferase activity normalized using control wells with virus and with cells alone.

Hemagglutination Inhibition Assay (HAI)

Serum samples were first treated with receptor destroying enzyme by incubation overnight at 37°C, and then incubated 30 min at 56°C. Sera were serially diluted, mixed with 8 HA units (HAU) of inactivated influenza virus (A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2)) and incubated for 30 min at room temperature prior to adding 0.7% turkey red blood cells. The highest serum dilution preventing hemagglutination was scored as the HAI titre.

RESULTS

Fabrication of dissolvable microneedle arrays

We developed a method for fabrication of DMN that will resolve drawbacks of the current methods. The proposed method (**Fig. 1b)** avoids the need for vacuum or centrifugation (**Fig. 1a)**, it is designed to be scalable to a production level and it does not waste or involve any re-use of materials.

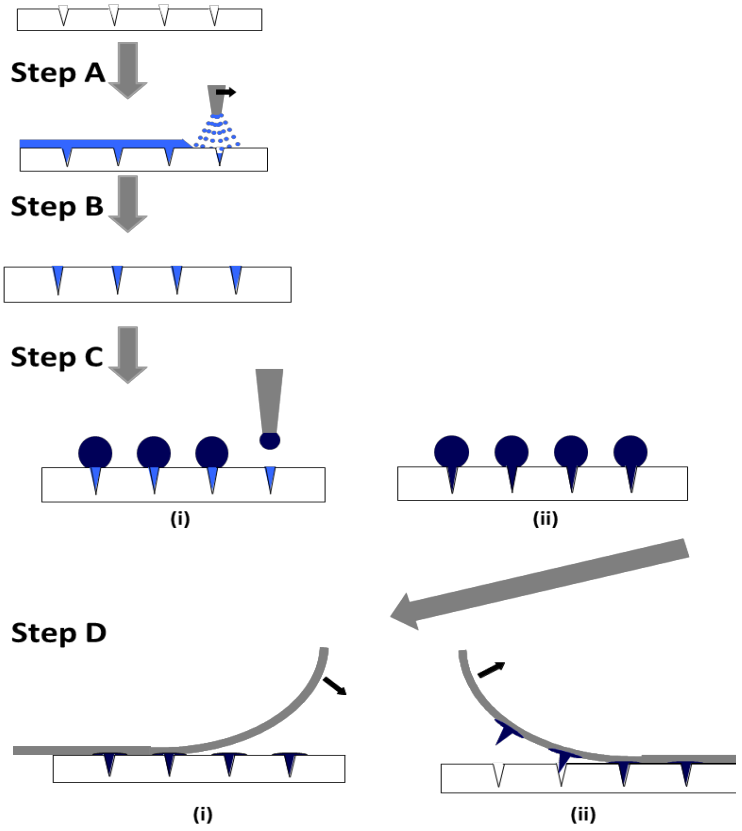
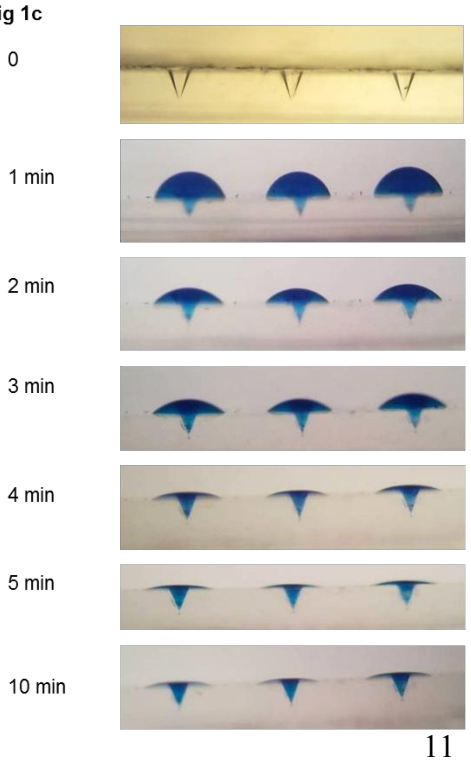


Fig. 1b

Moulds made from PDMS were first spray-filled with water [15] to fill cavities and remove air. Excess water was then removed from mould surface using a flat blade. A small amount of concentrated formulation (approximately 20 nL for 280 μm microneedles to 150 nL for 500 μm microneedles) was then dispensed directly on top of each microneedle well so that formulation and



water were in direct contact (**Supplementary Video 1)**. The hydrophobic nature of the PDMS mould prevented the formulation wetting the PDMS mould surface and retained the formulation over the water filled microdepression. A concentration gradient was immediately formed between the water in the microneedle mould and the formulation on top. Diffusion of water into the upper layer and formulation into the microneedle pore equilibrated the concentration in the two compartments (**Fig. 1c**). The diffusion efficiency of the concentrated formulation and water during the drying process was visualized in real time (**Fig. 1c**). The gradient was formed almost instantly as even after 1 min methylene blue dye contained in the upper drop was evenly distributed across the whole microneedle volume. As water evaporates the volume of the applied drop of formulation decreasesd leaving, at the end of the drying process, the amount of dried formulation sufficient to fill microneedle cavities completely. No vacuum or centrifugation was applied or was necessary during this diffusion process. The rate of diffusion of the active material into the microneedle pore can be affected by environmental temperature and humidity. For a 45% (w/v) trehalose formulation at ambient conditions, diffusion into the mould was complete after approximately 5 minutes (**Fig. 1c**). The importance of pre-filling with water is demonstrated by incomplete, stub-like structures being formed when this step is omitted (**Fig 1d)**.

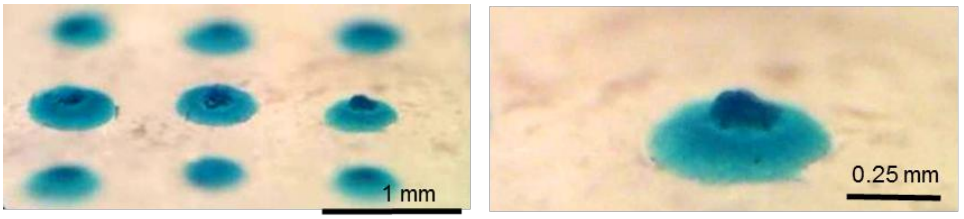


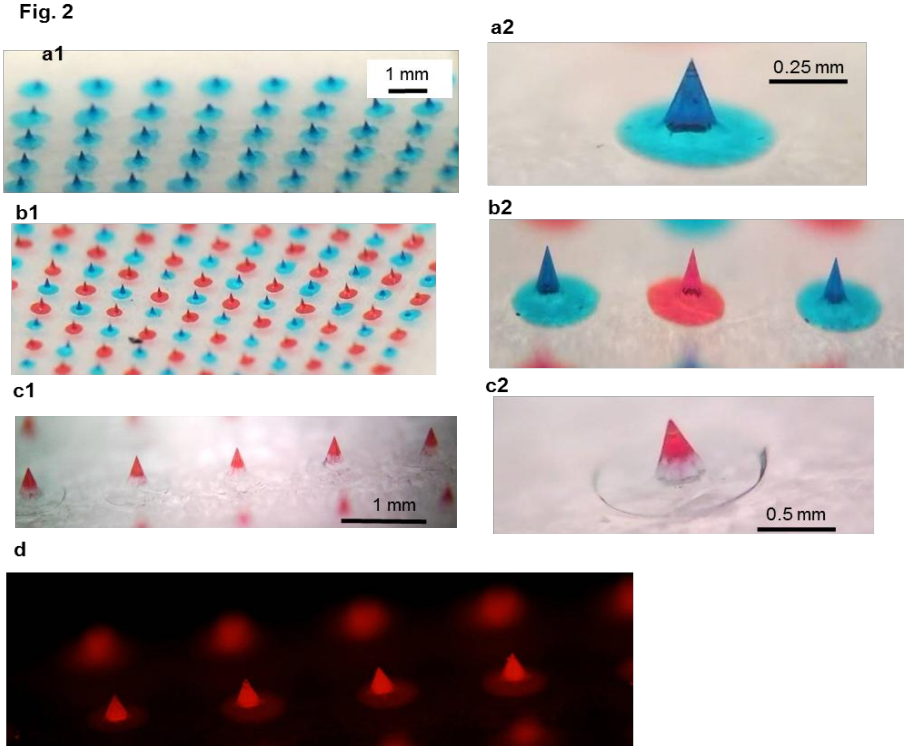
Fig 1d

After drying, adhesive backing tape was applied onto the PDMS mould and attached to the microneedles. The microneedles are pulled from the mould by lifting the adhesive tape, resulting in an array of DMN arranged on adhesive backing, ready for application onto skin (**Fig. 1**). No

additional backing layer is required. All of these process steps can be conducted in a continuous manufacturing mode. As microneedles in the described method are not formed in a batch mode but in a sequential mode, it is straightforward to make heterogeneous patches consisting of microneedles made of different formulations (**Fig 2**). Dissolvable microneedles with formulation concentrated in the microneedle tips can also be easily made by using a less concentrated formulation or dispensing a smaller drop (**Fig. 2c**). The overall result, when dried, is that there is a much lower amount of dried solids deposited into the mould. The remaining space of the microneedle is then filled with a second layer.

The next concern was that larger particles of interest present in the formulation (e.g. viral particles, long polymer chains) might not diffuse through the water-filled cavity as well as much smaller formulation excipients. This could result in non-homogeneous microneedles where active substances would be trapped in the microneedle bases while tips would be formed predominantly of excipients, resulting in sub-optimal delivery. This was addressed initially by using 100 nm fluorescent microspheres instead of methylene blue. Fluorescent microscopy of the formed microneedles shows that microspheres are uniformly distributed across the whole volume proving that concentration equilibration was effective even for large virus-size particles (**Fig. 2d**).

Fig. 2



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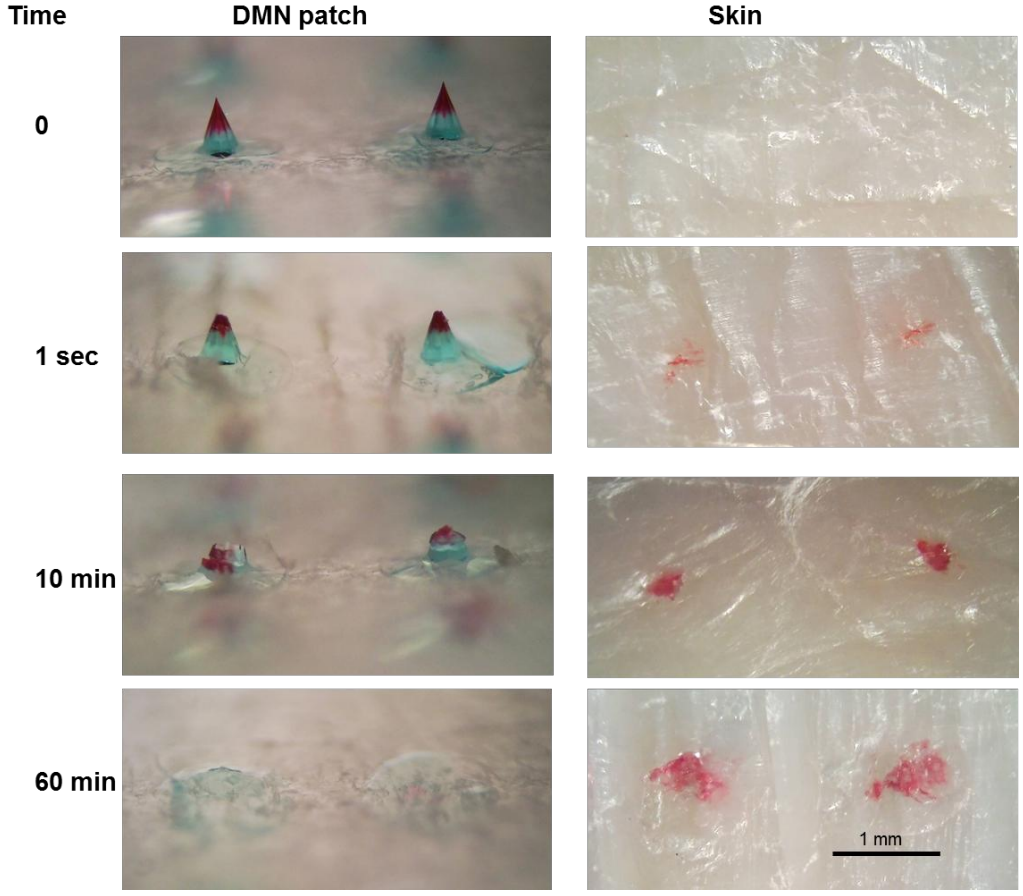
Functionality of DMN patches

A critical property of DMN patches is their capacity to insert into and dissolve in skin at the required rate. Ideally this process would be instantaneous for vaccines, to prevent re-use of patches, however, depending on the microneedle length, skin thickness and formulation release time, dissolution can range from few seconds to few hours or longer. We measured the kinetics of dissolution of our microneedles by insertion into cadaver pig skin. Test microneedles, consisting of tips made of trehalose with the addition of Congo red dye and a PVP base incorporating methylene blue, were inserted into pig skin and left for 1 s, 10 min and 60 minutes. While most of the formulation is released within the first few minutes, all the formulation is released in the skin between 10 minutes and one hour **(Fig. 3**).

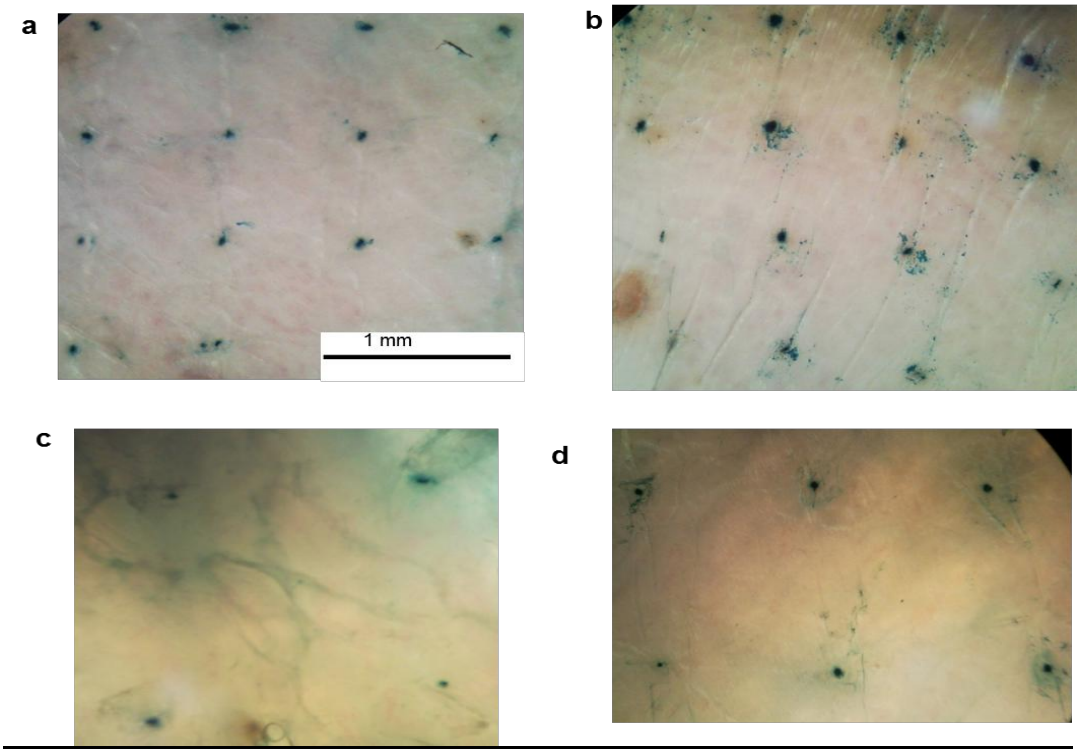
Fig. 3



Application of microneedles for transcutaneous delivery of live vaccines

Having defined this process for inert small molecules or nanoparticles, we then examined its suitability for incorporating labile vaccines using formulations previously identified for spray-coating these vaccines onto silicon microneedles [13, 24]. We tested the incorporation of recombinant adenovirus (AdV) and modified vaccinia virus Ankara (MVA) as these virus vectors are clinically relevant, yet they differ quite substantially in their biochemical and physical structure. By choosing appropriate trehalose-based formulations that did not degrade the vaccines in the liquid or solid state [13], both of these vaccines were successfully incorporated in DMN patches using this novel process. Patches containing either AdV or MVA expressing β-galactosidase were applied to freshly excised pig skin (**Fig. 4a, b**). Infection was achieved in both cases proving that viruses remained viable in microneedles, that microneedles penetrated the skin and that the delivered virus productively infected skin cells resulting in transgene expression. Infection was also obtained with microneedles with viruses embedded in the tips only (**Fig 4c, d).**

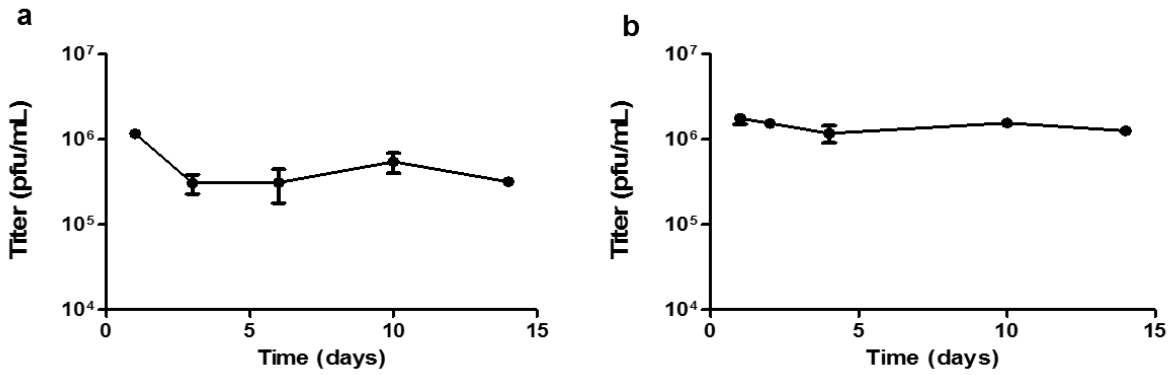
Fig. 4



Dissolvable microneedle patches stabilize vaccines outside cold chain conditions.

A motivation for developing the new method was to use microneedles as a vaccine stabilization as well as delivery platform. Stabilization of live viruses in a dried form and stable at ambient temperatures is a challenging task. Here we selected 50% trehalose (w/v) in water as the base formulation for AdV and a combination of 25% trehalose (w/v) and 7.5% PVA (w/v) solution in water for MVA. Results show that both viruses can be efficiently stabilized in DMN with a titer drop upon drying being less than 1 log unit over two weeks at ambient conditions (**Fig. 5**). It is important to note that most of the initial titer loss occurs during the initial drying period after which titer stabilises. Longer term stability studies of adenovirus are currently ongoing.

Fig. 5



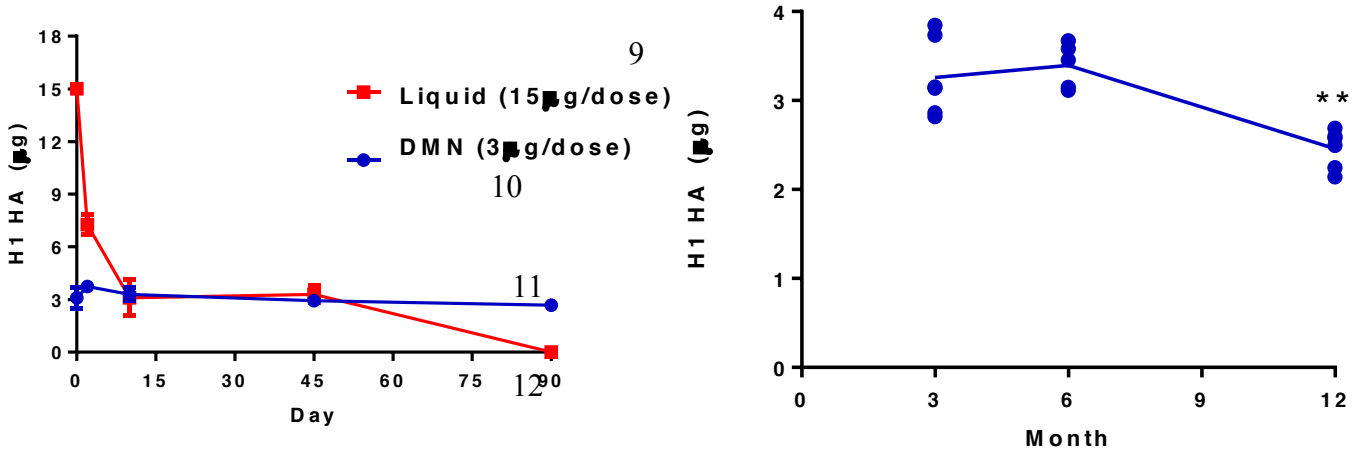
Long-term stability of clinically-used seasonal inactivated influenza vaccine in DMN

Next, we determined longer-term stability of a clinically licensed subunit vaccine when incorporated into DMN patches using our novel process. Trivalent inactivated influenza vaccine (TIV) from the 2011/2012 northern hemisphere season was mixed with a trehalose/PVA formulation (11% and 2.75% w/v respectively) and incorporated into DMN patches (500 μm tall microneedles). Similar to other studies [4, 25], we concentrated the vaccine to facilitate loading higher doses within a small volume of the microneedle patch. No deterioration in antigen activity was observed during the concentration and DMN fabrication process, as assessed by single radial immunodiffusion (SRID) [16, 17], (**Fig. S1**). The stability of 1.5 μg of the H1N1 and H3N2 hemagglutinin (HA) antigens per 1cm^2 DMN patch was compared to full dose (15 μg of each antigen) 2011/2012 seasonal TIV vaccine in pre-filled syringe at 40°C using SRID and looking at anti-HA IgG serum responses in mice for up to 1 year post-fabrication (**Fig 6**). A second study also assessed the stability of a lower antigen dose (0.3 μg of each antigen) at 1 year post-fabrication at accelerated conditions. Surprisingly, the A/Perth/16/2009 (H3N2) HA antigen demonstrated excellent stability in both the liquid and solid dosage forms (**Fig 6b**). Although the A/California/7/2009 (H1N1) HA antigen was labile in liquid form in the pre-filled syringe and had significantly degraded by day 10, this HA antigen was remarkably stable in the DMN patch and only began to decay between 6 and 12 months (**Fig 6a**). Immunogenicity studies supported the

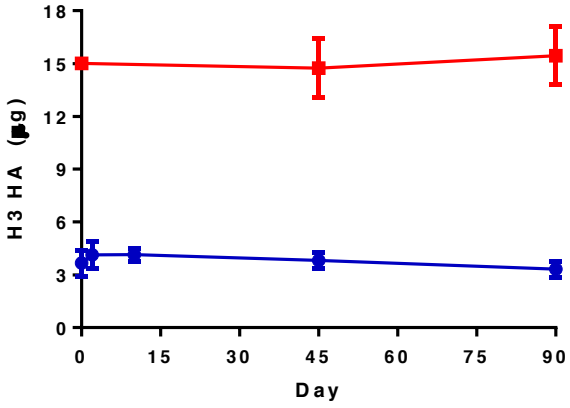
SRID stability results (**Fig 6 c,d**); immunization of mice with vaccine-loaded DMN patches that were stored for up to 1 year induced significantly greater anti-H1N1 and anti-H3N2 HA IgG compared to the stored liquid vaccine. To our knowledge, this level of stability has not previously been demonstrated at these accelerated conditions (40ºC) for any influenza vaccine stabilization technology. Therefore incorporation of a subunit vaccine into DMN using this novel fabrication process significantly enhanced stability out of cold chain.

Fig 6

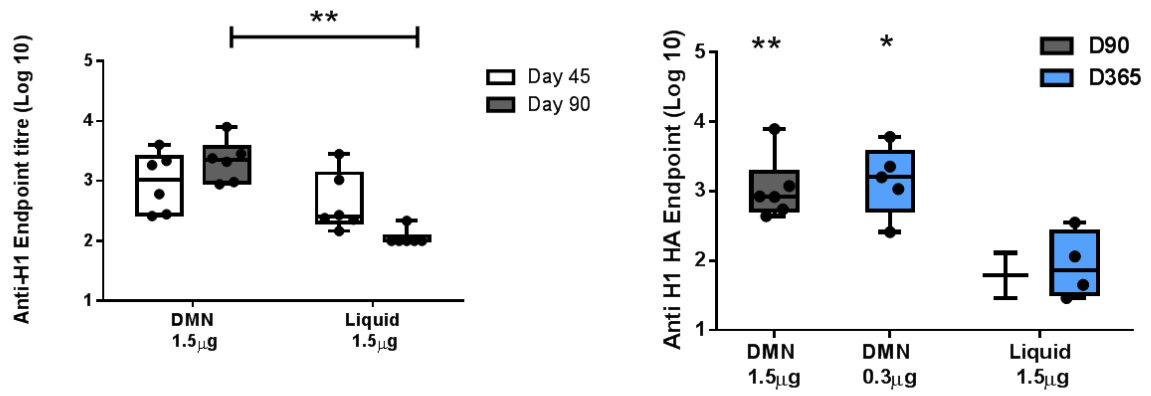
a



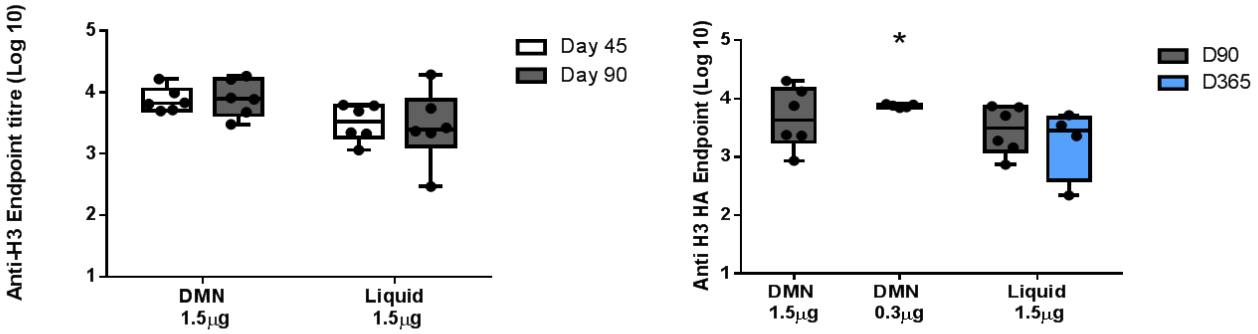
6b



6c


**
Anti-H1 Endpoint titre (Log 10)
5
4
3
2
1
DMN
1.5µg
Liquid
1.5µg
Day 45
Day 90
**
*
Anti H1 HA Endpoint (Log 10)
DMN
1.5µg
DMN
0.3µg
Liquid
1.5µg
D90
D365

6d


Anti-H3 Endpoint titre (Log 10)
5
4
3
2
1
DMN
1.5µg
Liquid
1.5µg
Day 45
Day 90
*
Anti H3 HA Endpoint (Log 10)
DMN
1.5µg
DMN
0.3µg
Liquid
1.5µg
D90
D365

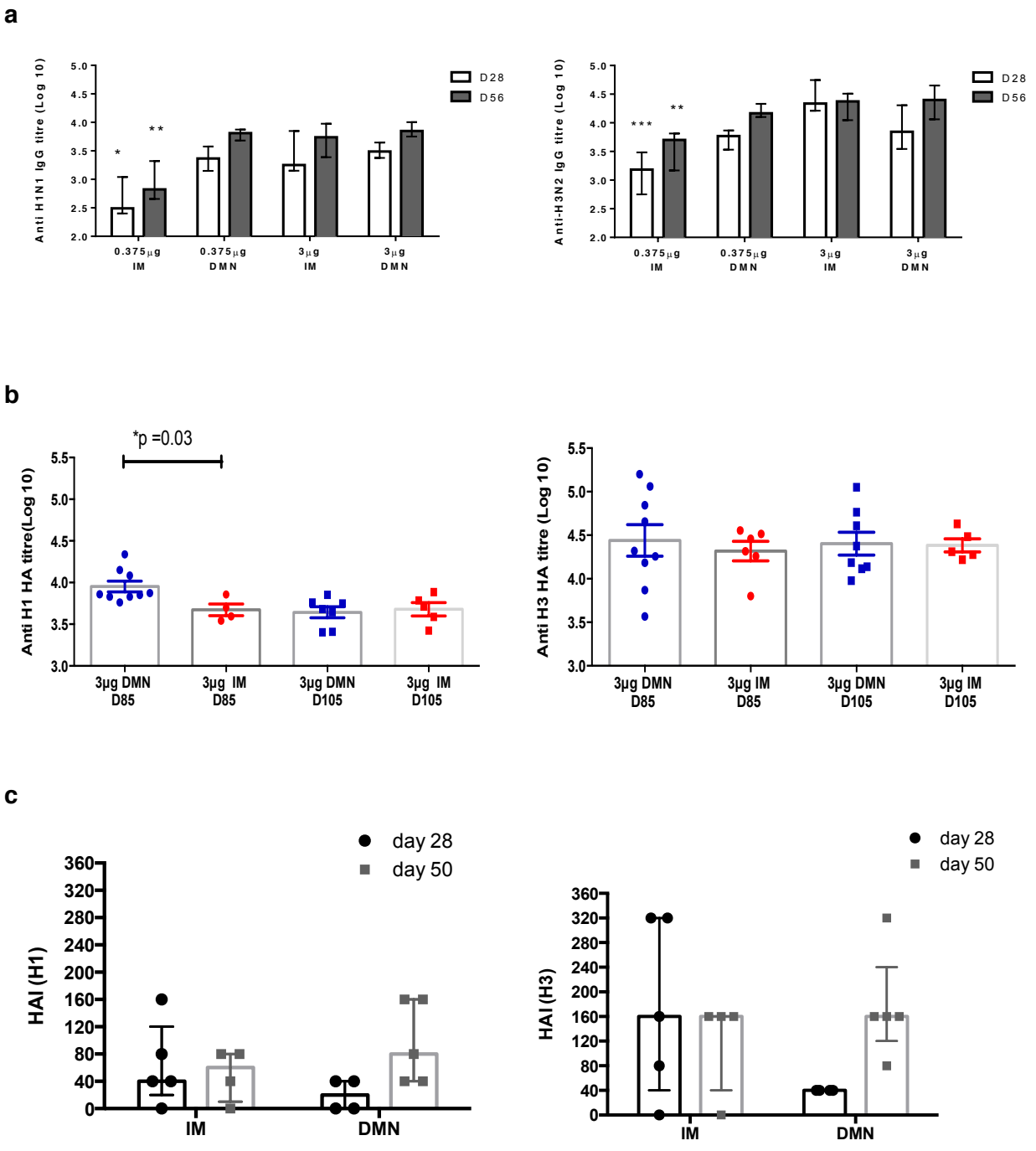
1

2

Immunogenicity of clinically-available seasonal inactivated influenza vaccine (TIV) delivered with DMN patches in mice

We next determined the immunogenicity of clinically-available, egg-derived, seasonal inactivated split trivalent influenza vaccine (TIV) freshly incorporated into DMN patches in mice. Previous studies with dissolvable microneedles and influenza vaccine have generally focused on whole inactivated monovalent virus or adjuvanted vaccines [8, 26-28]. As these vaccines are whole virus particles they are significantly more immunogenic compared to unadjuvanted TIV. Dose-sparing of influenza vaccine can be achieved by the intradermal route using hollow microneedles [29] and has been demonstrated in mice with a virus-like particle influenza vaccine or TIV-coated onto steel or silicon microneedles [30, 31]. Here, incorporation of the trivalent 2011/2012 seasonal TIV into DMN patches resulted in significant dose-sparing compared to IM administration. The IM route was chosen the vast majority of influenza vaccines are delivered by the IM route in the clinic; it also remains the standard route to which all other new influenza vaccine technologies, including microneedle delivery systems [4, 25, 32, 33] are compared as there is a wealth of information and understanding in both pre-clinical models and clinical trials. A single immunization with the lower dose TIV-loaded DMN patches (0.375 μg of each HA) induced an anti-HA IgG response that was significantly higher than responses induced by IM immunization, even at 12 weeks post-immunization (**Figure 7**). Finally, immunization using the higher dose (3 μg HA) of vaccine in DMN patches induced a significantly more durable anti-H1N1 HA IgG serum response 12 weeks after a single immunization **(Fig 7B).** The IgG response to the H3N2 HA was also higher in some, but not all mice in the DMN group. In a separate study using a different DMN formulation of 12.5% (w/v) trehalose and 5% (w/v) PVA, examination of hemagglutination inhibition titres (HAI), which measures antibody binding to the HA head region that mediates hemagglutination of red blood cells, demonstrated that there was no significant differences in HAI, however there was a delay in HAI induction in the DMN-treated mice compared to IM injection (**Fig. 7C**).

Figure 7

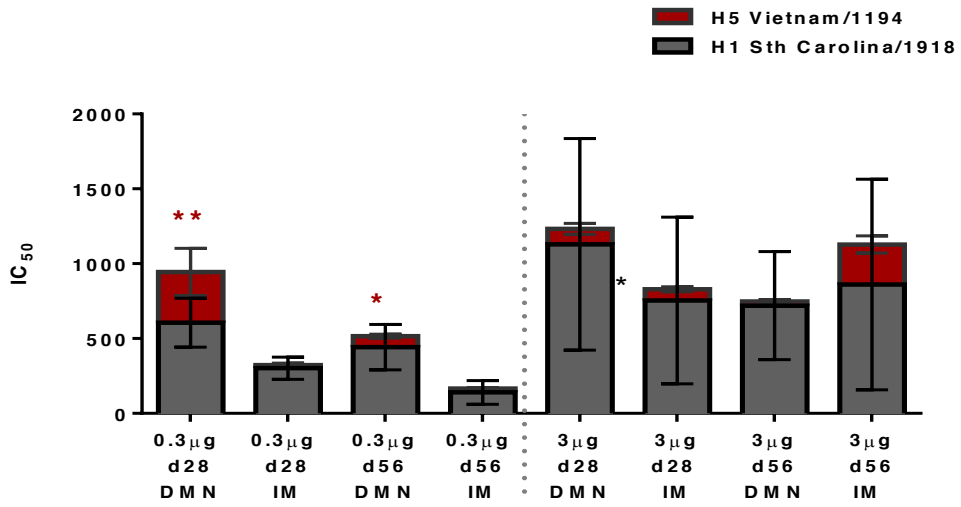


1 Induction of broadly neutralising antibodies by influenza vaccines is a highly sought after goal
2 [34]. To evaluate the breadth of neutralization, sera were analyzed for their ability to neutralize

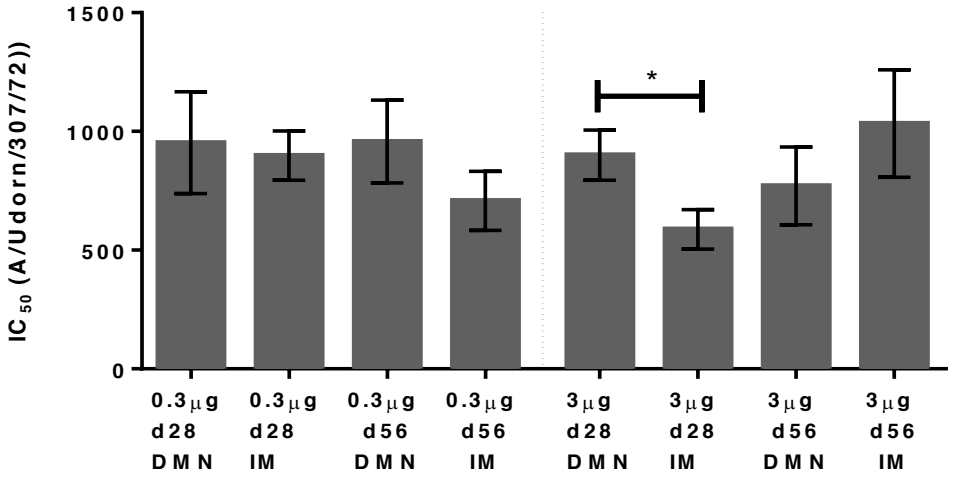
heterologous and heterosubtypic group 1 and 2 influenza strains using a pseudotype lentiviral assay (PPV) [35]. The HA antigens from A/South Carolina/1/1918 (H1N1) and A/Vietnam/1203/2004 (H5N1) have 86% and 65% identity to the H1N1 strain included in the vaccine respectively, and A/Udorn/307/1972 (H3N2) has 88% homology to the vaccine H3N2 HA antigen. Surprisingly a significant increase in the magnitude and duration of heterosubtypic immunity, to the H5N1 HA, was induced when a low dose of vaccine was delivered by DMN compared to IM (**Fig. 8**). The A/South Carolina/1/1918 response in the high dose DMN group was also significantly higher than the corresponding IM group at week 4. We then investigated if the significant increase in heterosubtypic immunity induced in low dose DMN group was due to neutralizing antibodies that recognized the stalk region of the HA antigen in the low dose vaccine groups using a chimeric HA antigen consisting of a head region from A/duck/Memphis/546/1974 (H11N9) HA and a H1 stalk from A/South Carolina/1/1918 (H1N1). A response to the stalk of H1 HA was deemed to be present if the IC_{50} to the chimeric antigen was greater than that against the H11 HA (**Table 1**). Using this PPV neutralization assay, a strong focusing of the anti-HA H1N1 response to the stalk region of HA was observed in the low dose IM group 8 weeks post-immunization, with little or no NAb detectable to the head regions of the tested heterologous H1 or heterosubtypic H5 HA antigens; this has not been previously demonstrated. In contrast, immunization using low dose vaccine in DMN patches expanded the antibody repertoire: one month post-immunization neutralizing antibodies (NAb) against the head regions of both H1 and H5 HA antigens predominated, with little anti-stalk NAb response being observed. At 2 months post-immunization with DMN a broad distribution of neutralizing antibodies was evident, with NAb to non-stalk regions of A/South Carolina/1/1918 H1N1 predominating over NAb to H5 and to the stalk region (**Fig. 8** and **Table** 1). Therefore DMN-based immunization induced a broad antibody neutralizing antibody range that recognized heterosubtypic influenza virus strains and the non-stalk regions of the HA antigens.

Figure 8:

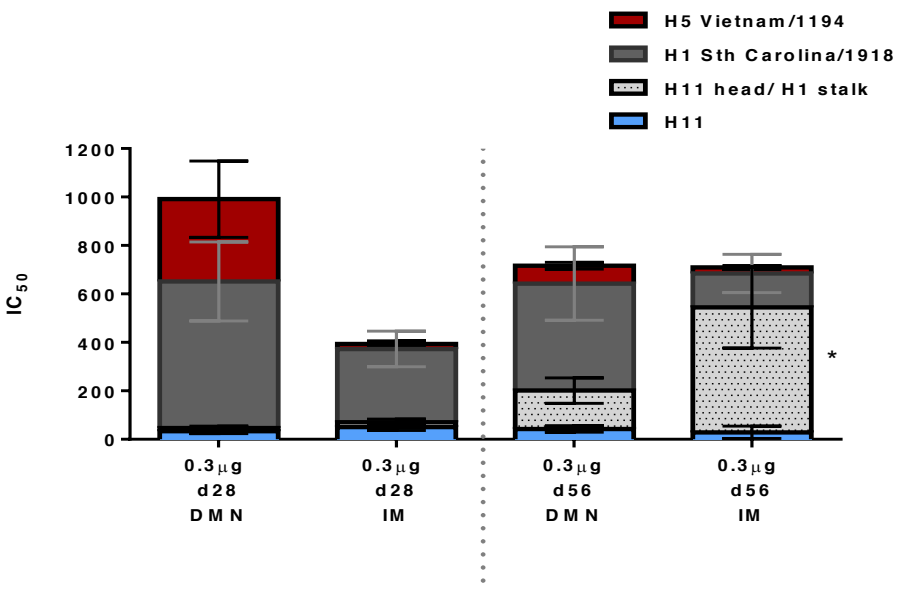
a


H5 Vietnam/1194
H1 Sth Carolina/1918
IC50
2000
1500
1000
500
0
**
*
*
0.3µg d28 DMN
0.3µg d28 IM
0.3µg d56 DMN
0.3µg d56 IM
3µg d28 DMN
3µg d28 IM
3µg d56 DMN
3µg d56 IM

b


IC50 (A/Udorn/307/72))
1500
1000
500
0
*
0.3µg d28 DMN
0.3µg d28 IM
0.3µg d56 DMN
0.3µg d56 IM
3µg d28 DMN
3µg d28 IM
3µg d56 DMN
3µg d56 IM

c



DISCUSSION

Dissolvable microneedles offer several advantages to deliver vaccine safely and effectively and their successful development as needle-free, easy-to-administer, *in vivo* reconstitution, stable and cheap systems would address several logistic obstacles that are currently preventing immunization programmes from reaching their full potential [1, 2]. However, current fabrication methods are constrained by a number of obstacles, including the use of semi-continuous steps, such as centrifugation or vacuuming, that may not be easily scalable [4, 36]. Our over-arching aims were to develop a process to make dissolvable microneedle patches which overcomes this translational hurdle and to demonstrate the utility of this stabilized vaccine delivery platform at a pre-clinical level. Early methods of making DMN patches involved applying excess formulation to the mould, vacuuming the mould to pull the mixture into the microneedle pores and then gently removing the solution that had “puddled” on the surface of the mould with a micropipette [36].

Although this method and similar, batch-based centrifugation-based methods [4, 25] is useful for lab-based investigation of the technology, it may not be optimal at large scale. Studies have suggested that microneedle patch-based immunization can lead to dose-sparing in mice [30, 31], however, such an advantage will be lost at the microneedle fill-finish stage when significant losses are incurred using these methods. We therefore defined a novel fabrication process to overcome these issues and reduce the barriers to translation. Specifically, we created a simple process using pharmaceutically-relevant technologies that have been previously used at a high manufacturing scale, which did not result in waste of the active material and are amenable to continuous manufacturing [37]. The method is based on prefilling the moulds with water and subsequent delivery of the formulation on top of wells, after which the diffusion process equilibrates the formulation concentration in the whole microneedle volume. By precisely dispensing the vaccine formulation onto pre-filled moulds no material was wasted. Subsequent drying of the formulation delivered to the mould results in the dry and rigid microneedles that can be directly transferred to adhesive film in a form ready for application on the skin. As a result a further simplification over the current methods is that no additional backing layer anchoring the microneedles is needed.

Another disadvantage of current methods for production of DMN is that the whole array effectively has to be made of the same type of microneedles (filled with the same formulation). The described method is also readily amenable to producing heterogeneous arrays containing two or more types of microneedles. This permits, for example, separation of incompatible materials or localization of microneedles that dissolve at different rates (permitting *in vivo* prime-boosting), containing different antigens or doses or requiring different stabilising excipients. Thus, our process demonstrates strong potential to be scalable to manufacturing level and administration of different vaccines or delivery kinetics in the same patch. It also overcomes issues such as waste and potential GMP issues associated with reuse of excess formulation

during manufacture. In preliminary studies (data not shown) full human doses of TIV vaccine were easily incorporated into two 1cm^2 patches. However, we focused on understanding the dose-response relationship at lower doses of vaccine per patch in the linear range of this dose curve. Furthermore, given the current discussion in the field on the effect of high concentration of full vaccine doses on antigen integrity [4, 25], incorporating a full human vaccine dose across a wider area than 1-2 cm^2 may be required for optimal antigen stability in the future.

Recombinant live viral vector vaccines are the subject of intensive research. Some of the most promising vaccine candidates for diseases such as Ebola virus malaria, HIV and tuberculosis as well as for certain cancer treatments [38-41] utilize these platforms. If efficacy of these vaccines is demonstrated, a critical issue that will need to then be overcome is the financial and logistic cost surrounding cold chain distribution and/or long-term stockpiling using non-cold chain storage systems. Stabilization of recombinant live viruses can be challenging, however, virus infectivity assays represent a sensitive screen for identifying formulations that will retain vaccine viability. Suitable formulations for stabilizing vaccine in DMN patches must be capable of preserving vaccine integrity during microneedle fabrication, upon drying and during subsequent storage. Many of the reported formulations containing viscosity enhancers such as CMC or surfactants [26, 28, 42-45] were not suitable in our work as we previously found them harmful for AdV and MVA [13]. Addition of other excipients may further enhance physicochemical and physical characteristics of microneedles, such as equilibrium moisture content, dissolution kinetics, mechanical strength or improve vaccine stability. In the case of MVA and the subunit TIV vaccine we found that addition of PVA into trehalose solution retains vaccine integrity upon drying and produces a highly stable vaccine product. Short-term stability of AdV and MVA vectors embedded in our microneedles and kept at ambient temperature over 14 days showed that viruses were well preserved in the dry form and initial losses are comparable or better than freeze drying methods [46]. In longer-term stability studies with TIV, a drop in H1N1 HA activity

was only observed between 6 and 12 months storage at 40°C. Indeed, mice immunized with a lower dose vaccine-DMN patch (0.3 μg/patch) that had been stored at 40ºC for 1 year responded with equivalent IgG responses [28] to those achieved with higher dose fresh patches (as seen in **Fig 6**). In contrast, we observed rapid degradation of the H1N1 HA, but not the H3N2 HA in the pre-filled syringe, with >25% degradation of the H1N1 HA occurring within 10 days. An 80% decrease in H1N1 HA content in the liquid vaccine was observed at day 45, however, the remaining dose (3μg) was immunogenic in mice, in keeping with the dose response experiments (**Fig 6**). Due to the dose-sparing nature of microneedle patches, a loss in HA content may not be evident in *in vivo* immunogenicity tests, therefore regulatory-approved SRID assays were used as a main stability assay. However, the immunogenicity data in these stability studies agrees with the known differences in the structural stability of these HA antigens from different influenza strains [47]. To our knowledge, such long-term stability of influenza vaccine in microneedle patches at such elevated temperatures has not been demonstrated. Recently, a dextran-based influenza vaccine-loaded DMN system [25] or hyaluronic acid-based microneedle patch system [48] demonstrated lower stability compared to our system when samples were stored at ambient (25ºC) or accelerated (40ºC) temperatures and tested using SRID or *in vivo*. However, a monovalent influenza vaccine demonstrated promising stability at 25ºC for 3 months [32] and using in-house ELISA-based assays, other formulations have been suggested for stabilizing influenza vaccines in microneedle structures [49] Other efforts that incorporated TIV onto transcutaneous patches were also less successful at elevated temperatures; however the relative stability of the H1N1 compared to H3N2 antigens was also observed in this study [50]. Our study therefore further confirms the capacity for the described formulations and fabrication process to contribute to protein stabilization and retention of antigen integrity. These findings, along with the other advantages of the fabrication method, demonstrate the potential of this dissolvable microneedle patch technology for stabilizing vaccine out of cold chain. Furthermore, the cubic volume and weight of a final, packaged single-dose microneedle patch format will likely

be significantly lower than current single or multi-dose vaccine vials thereby further permitting more cost-effective distribution of vaccines.

Although we focused on solving questions relating to the manufacturability of dissolvable microneedle patches, clinical usability of a final product will also need to be addressed in future studies. To enhance the cost-efficiency [51] and ease-of-use of this patch, we aim to develop a patch that does not require an applicator device. Our dissolvable microneedles have an octagonal cone shape with an ultra-sharp tip (approximately 50 nm) [52] and are mounted on flexible adhesive tape, compared to foam-based backing layers [53]. These dissolvable microneedles are mechanically strong enough to consistently penetrate murine and porcine skin using thumb pressure and without the need for an applicator. It is likely that the microneedle base will not remain embedded in the skin due to the skin's elasticity [54]. Hence, formulation contained in the microneedle base may not be delivered but would rather remain either on the array or unused on the skin surface therefore necessitating DMN with vaccine in the tip only to be used. We demonstrated that live vaccine-loaded DMN that were 280μm or 500μm tall with vaccine throughout the microneedle or only in the tip successfully penetrated into porcine skin and delivered live virus. Successful skin transfection by live virus vaccines delivered by these DMN patches confirms that virus is well preserved in the microneedles, that microneedles penetrate the skin and that skin cells were successfully transfected and produced β-galactosidase. Similar to other microneedle technologies [32, 55, 56] it is unlikely that 100% of the vaccine was delivered into the skin, particularly when the vaccine is distributed throughout the microneedle. This suggests that even greater dose-sparing may have been achieved in this mouse model due to sub-optimal vaccine delivery efficiency. However, similar to these other publications [8], concentration of the active material into the tip of the microneedles, or increased dose adjustment [55] should enhance the efficiency of delivery, which will likely be more important in humans, compared to the thinner skin of mice. Microneedles with the formulation in

the whole microneedle volume (as used in these immunogenicity studies) could deliver vaccine components into both epidermis and dermis layers while tip-only microneedles could target dermis layer only. This could potentially result in markedly different responses [57]. In our immunogenicity studies we used vaccine distributed throughout the microneedle that likely delivers vaccine to murine epidermis and dermis. The type and breadth of the induced immune response induced may also be affected by the use of an applicator and the amount of force and stress experienced by the skin during this process [25], the formulation used, its dissolution kinetics and the location of antigen in the skin. These parameters should thus be taken into account when comparing immunity induced by different microneedle technologies.

An aim in the influenza vaccine field is to develop a universal vaccine that protects against a wide range of seasonal and newly emerging influenza virus strains. Substantial research demonstrates that antibody responses to the conserved stalk region of HA provide broadly cross-reactive neutralizing antibodies (NAb) [58-60]. A broadly neutralizing vaccine could function by potently inducing cross-reactive anti-stalk antibodies, however such a vaccine remains elusive. Alternatively, as is the case with influenza vaccine containing MF59 adjuvant, broadly neutralizing responses could be represented by antibodies that recognize a wider spread of HA epitopes, predominantly located in the HA1, head region, of the HA antigen [61, 62]. We initially hypothesized that the higher anti-H5 HA responses in the low dose DMN groups would associate with higher responses against the H1 HA stalk. However, we instead observed a more diverse profile of serum antibodies that recognized and neutralized heterologous and heterosubtypic HA antigens. This has not been previously demonstrated for microneedle-based immunization. It suggests that, compared to IM administration, vaccine delivery using these dissolvable microneedle patches permits expansion, differentiation and survival of multiple B cell clones in germinal centres, which recognize multiple epitopes in the HA antigen, few of which are in the stalk region of the HA antigen, similar to the action of MF59 adjuvant in humans [61,

62]. A more diverse B cell profile may also explain lower HAI titers at day 28 in the DMN-treated group. It is possible that at this time, HAI-producing B cells are not as frequent in DMN compared to IM treated animals. HAI is considered a correlate of protection for IM-administered TIV vaccines. Induction of lower HAI at early time points could suggest delayed vaccine efficacy if this assay was solely used as an efficacy marker. However, similar to intranasal live attenuated influenza vaccines, reduced or absent HAI may not correlate with disease susceptibility and detection of other immune mechanisms, including neutralizing antibodies, may be required to provide more appropriate correlates for vaccine efficacy in suitable pre-clinical and clinical studies, Further studies are required in a suitable influenza-primed animal model to determine if these TIV vaccine-loaded dissolvable microneedle patches can induce greater heterosubtypic protection compared to IM administration. We previously demonstrated that, unlike systemic immunization, silicon microneedle-mediated vaccination did not induce inflammatory responses at the site of immunization or in draining lymph nodes [19, 20]. We hypothesize that an increase in B cell diversity that are induced by vaccine-loaded DMN patches relates to differences in the cytokine environment that may be induced using DMN patches compared to IM vaccine administration. Further work is required to examine this hypothesis. Overall, our findings demonstrate that incorporation of a seasonal influenza vaccine into dissolvable microneedle patches significantly alters the phenotype of the humoral response compared to intramuscular delivery. This offers a novel method of inducing broad influenza-specific immunity independently of adjuvant utilization.

In summary, novel DMN fabrication methods were developed which overcome drawbacks of the current approaches as they offer simple and scalable production techniques that are designed for GMP-compliance. The described approach has the unique feature that heterogeneous arrays containing more than one type of microneedle on the same DMN patch may be easily prepared. Vaccines incorporated into DMN patches using these methods were significantly stabilized out of

cold chain and were delivered transcutaneoulsy. Significantly, a broader humoral response was induced using these dissolvable microneedle patches and significant vaccine stability was achieved. This study supports the further development of microneedle technology for vaccination purposes.

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FIGURE LEGENDS

Figure. 1. Schematic diagrams of dissolvable microneedle array fabrication processes. (a) Conventional fabrication process. **(b)** Novel fabrication process. **Step A:** Water was sprayed over a PDMS mold to fill the microneedle cavities. **Step B**: Excess water was removed from the surface using a flat blade. **Step C**: (i) The concentrated formulation was applied directly on top of microneedle cavities. (ii) The drug solution in the upper formulation diffused and equilibrated in the microneedle mould as the result of gradient formed between highly concentrated formulation and water in the cavities. (iii) The drug solution dried. **Step D**: Flexible adhesive tape was (i) applied on top of the mold to adhere to needle bases and (ii) lifted giving an array of drug-filled DMN ready for application. **(c)** Drying of drug formulation dropped on PDMS mould. Formulation consisting of 45% trehalose (w/v) and methylene blue delivered on top of PDMS mould diffuses into the mould in approximately 5 min at ambient conditions. First picture (1 min) also shows that diffusion of formulation from upper bulb into microneedle cavity is complete even after 1 min (microneedle tips are blue confirming that diffusion equilibrated concentration in the bulb and microneedle cavity). **(d**) Incomplete microneedles formed if prefilling of 280 μm tall microneedle cavities with water was omitted from fabrication procedure (D1-D2).

Figure 2. Examples of dissolvable microneedle patches. Homogeneous microneedle array (**a1**) and individual needle (**a2**). Heterogeneous array containing microneedles made of two different formulations (**b1**) and magnified part (**b2)**. Microneedle array with formulation concentrated in the needle tips with transparent PVP base (**c1-c2**). Fluorescent microscope image of microneedle array with 280 μm microneedles fabricated with 0.1 μm OD red fluorescent microspheres and trehalose as excipient (**d**). Fluorescence is equally distributed throughout the needles thus proving that diffusion of large virus-size particles was equal to diffusion of much smaller excipient molecules. Equal diffusion rates ensure the needle homogeneity upon drying (**d)**.

Figure 3. Kinetics of dissolution of DMN with formulation concentrated in the microneedle tips. Arrays with 500 μm tall needles were fabricated with tips made of trehalose with the addition of

Congo red dye and base made of PVP with the addition of methylene blue dye. Arrays were then applied onto cadaver pig skin and left for 1 s, 10 min and 60 min after which they were imaged using microscope.

Figure 4. Skin-transfection using dissolvable microneedle patches with AdV-β-galactosidase or MVA β-galactosidase throughout the entire microneedle (**a, b**) or distributed in the tip only (**c, d).** Microneedle arrays with 280 μm long microneedles containing either AdV β-gal (**a)** or MVA β-gal (**b**) or 500 μm long microneedles (**c, d**) were applied onto freshly excised pig skin and later examined for β-galactosidase expression at 24 hours.

Figure 5. Stability of AdV and MVA embedded in microneedle patches during 14 days at ambient temperature. AdHu5-mCherry **(a)** or MVA-RFP (**b**) were embedded in DMN made of trehalose (AdV) or trehalose/PVA (MVA) and left at ambient temperature for 14 days. Y-axis represents titer in $\log(PFU)_{eq}$ units for AdV and MVA with error bars showing SD.

Figure 6. One-year stability of influenza vaccine in microneedle patches. Stability of HA antigen as assessed by content (**a, b**) and immunogenicity (**c, d**) when incorporated in DMN patches or as a liquid in pre-filled syringes, over time at ICH-recommended accelerated conditions (stability chamber set to 40°C, 75% RH). DMN patch composition was 1.5 μg or 0.3 μg HA in 11% (w/v) trehalose and 2.75% (w/v) PVA per dose or a full dose (15 μg) in liquid. **(a)** A/California/7/2009 (H1N1) and (**b**) A/Perth/16/2009 (H3N2) HA content as assessed by SRID. Immunogenicity of influenza vaccine incorporated in DMN patches or in liquid vaccine to 3 months (left) or 1 year (right). **(c)** A/California/7/2009 (H1N1) and (**d**) A/Perth/16/2009 (H3N2) HA-specific serum IgG responses. BALB/c mice were immunized by the intramuscular route for the liquid vaccine or using TIV-loaded DMN patches. Single patches containing 1.5 μg HA in DMN patches (day 45, day 90) or 0.3 μg HA (day 365) were applied to an ear of each mouse. Total IgG titers in blood were measured to recombinant HA at 4 weeks after immunization. * p , 0.05, ** p , 0.01, ***p , 0.001 compared to

liquid vaccine at the same time point by t-test. The median and interquartile range are represented by the bar and whisker plots, with individual responses (n = 6) shown.

Figure 7 Anti-Hemagglutinin responses. (a) Serum IgG responses to hemagglutinin A/California/7/2009 pdm09 (H1N1) (left) and A/Victoria/210/2009 (H3N2) (right) responses after a single immunisation of mice on day 0 with 0.375 µg HA or 3 µg HA of TIV via DMN or IM routes. Median responses with interquartile range. *=p <0.05 **p<0.01***p<0.001 for 0.375 µg IM group compared to all other groups (n = 6 per group). (**b**) In a separate study, the durability of the anti-HA IgG response was assessed at 85 or 105 days after a single immunization with 3µg of each HA using DMN or the IM route. *=p <0.05 for DMN compared to IM at day 85 by t-test. (**c**) Hemagglutination inhibition (HI) titres at four and eight weeks post prime. HI titres against H1A/California/2009 (left) and H3 A/Victoria/207 (right) strains at 4 and 8 weeks post prime with a single immunisation of 3µg HA in 4-6 week old female BALB/c Mice (n = 4 -5 group). Bars represent median values with interquartile range.

Figure 8. TIV-loaded microneedle patches induce broadly neutralising antibodies. Pseuotyped neutralization assay to measure the neutralizing antibody response in mice immunized with TIV incorporated in DMN patches or administered by the IM route. The neutralizing antibody response in mice against (**a)** influenza group 1 viruses A/South Carolina/1/1918 (H1N1) and A/Vietnam/1203/2004 (H5N1); (**b)** HA from the group 2 virus A/Udorn/307/1972 (H3N2) HA-pseudotyped lentivirus reporters after immunization with or 3 µg or 0.3 µg HA. **(c)** Neutralizing antibodies to the chimeric HA, with the responses against the other group 1 viruses (displayed in (A)) for comparison, at 28 or 56 days after a single immunization with 0.3 µg HA using DMN patches or the IM route . *=p <0.05 **p<0.01 for DMN compared to IM group for the same dose, by one-way ANOVA (n = 5 per group).

Figure S1 Antigen stability post fabrication. Single radial immunodiffusion gels demonstrating that fabrication has no detectable effect on hemagglutinin A/California H1N1 (left) or A/Victoria

H3N2 (right). Individual samples were added neat (1.0) or diluted to 0.75, 0.5, 0.25 of the neat concentration, as indicated, in duplicate. Licensed trivalent influenza virus vaccine (Fluarix), was concentrated 7.9 fold prior to formulation (Fluarix conc. 7.9x) to ensure sufficient vaccine loading in patch. Reference antigen (Ref antigen) from NIBSC, dissolvable microneedle patches (P1-6) and liquid formulation used to fabricate DMN (Liq. Form) as well as concentrated vaccine were dissolved and diluted to yield a theoretical content of 3 μg hemagglutinin as the neat solution

Table 1. Neutralising antibody responses to group 1 HA antigens. Neutralizing antibodies to the chimeric HA, with the responses against the other tested group 1 viruses for comparison, at 28 or 56 days after a single immunization with 0.3 μg HA using DMN patches or the IM route. A response to the stalk region of A/South Carolina/1/1918 (H1N1) HA is deemed positive if the IC_{50} to the chimeric, H11/H1 head/stalk antigen is greater than that observed to the A/duck/Memphis/546/1974 (H11N9) HA. Shading highlights responses that are greater than the median response observed across all samples. *; indicates that the response to H11N9 is greater than the response H1N1 HA.

AUTHOR CONTRIBUTION

Conceived and designed the experiments: AV, EAA and ACM. Performed the experiments: AV, EAA, FF and ACM. Analyzed the data: AV, EAA, FF, NJT, AMC and ACM. Contributed reagents/materials/analysis tools: AMC. Wrote the paper: AV and ACM. All authors reviewed the final version of manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests. AV, AMC, ACM are named inventors on patent applications covering microneedle-mediated drug and vaccine delivery.

Table 1. Neutralising antibody responses to group 1 HA antigens

	H11	Chimeric H11-Head /H1 Stalk	H1	H5	Stalk Response??	Ranking
Median IC_{50}		*55*	*293*	*39*		
Day 28						
DMN#1	49	0	41	163	NO	H5 > H1
DMN#2	23	0	1036	471	NO	H1 > H5
DMN#3	40	0	68	9	NO	Negative
DMN#4	3	32	482	0	YES	H1 >Stalk
DMN#5	0	57	847	180	YES	H1 >H5 > Stalk
DMN#6	40	0	658	356	NO	H1 > H5
DMN#7	80	0	1105	1199	NO	H1 = H5
IM #1	1	77	293		YES	H1 > Stalk
IM #2	54	26	316	0	NO	H1
IM #3	45	15	216	25	NO	weak H1
IM #4	50	3	148	55	NO	H5, weak H1
IM #5	107	0	648		NO	H1
IM #6	47	0	191	1	NO	weak H1
Day 56						
DMN#1	56	300	37	39	*	Stalk > H1
DMN#2	98	33	1105	97	NO	H1 > H5
DMN#3	2	334	538	32	YES	H1 > stalk,
DMN#4	29	127	460	95	YES	H1 > stalk > H5
DMN#5	32	55	317	65	YES	H1 > stalk, H5
DMN#6	41	100	193	113	YES	H1, stalk, H5
IM #1	13	223	14	63	???	Stalk > H5
IM #2	2	467	37	8	YES	Stalk
IM #3	0	61	56	25	YES	Stalk
IM #4	0	475	144	20	YES	Stalk
IM #5	0	1248	526	24	YES	Stalk > H1
IM #6	157	617	66	7	*	Stalk

Figure(s)

Fig. 1a

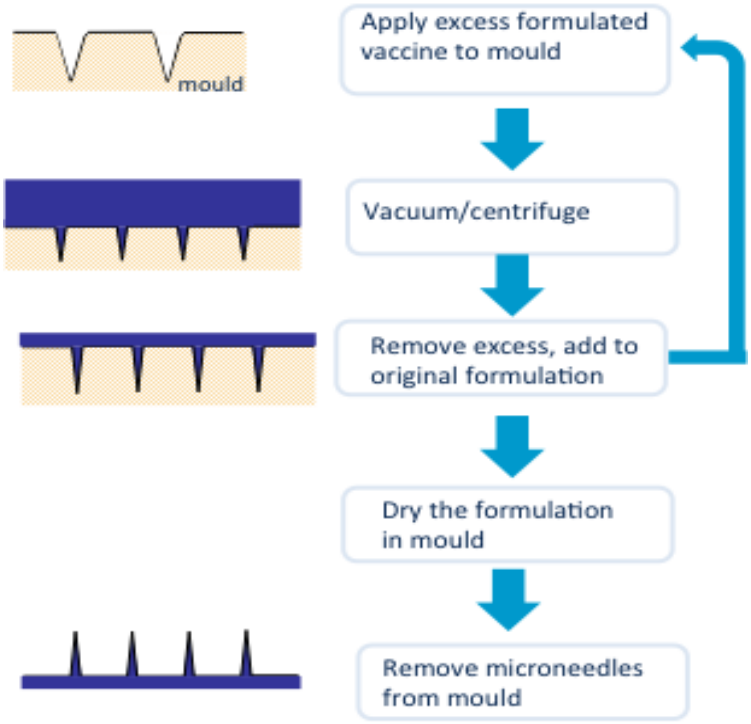

Figure 1b

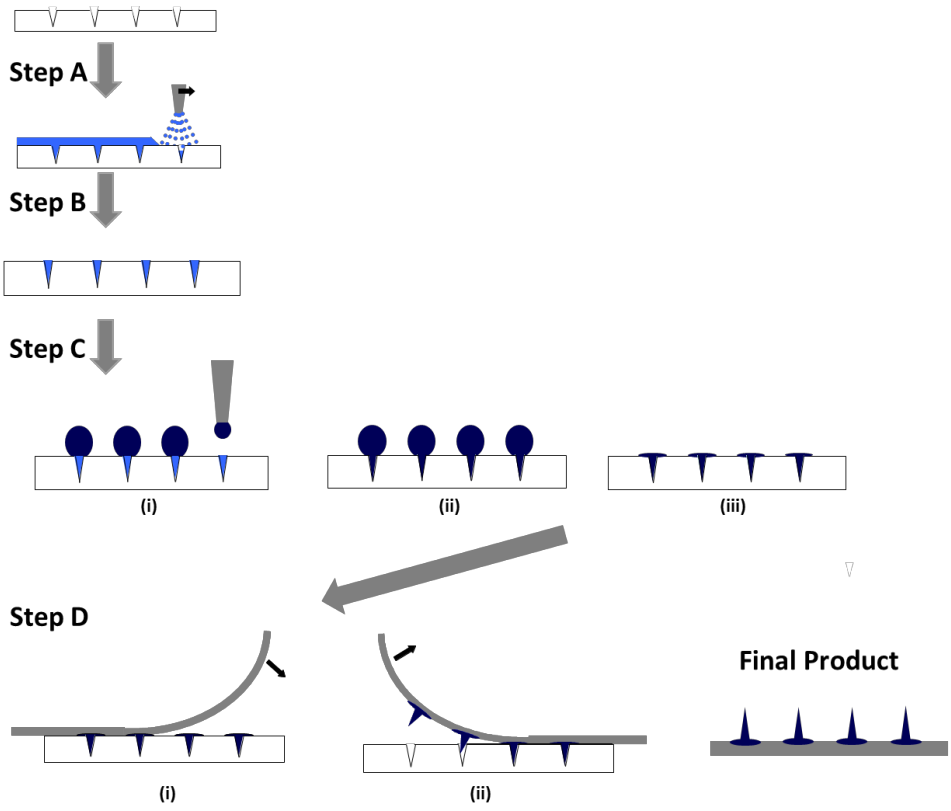

Fig. 1b

Figure 1c.

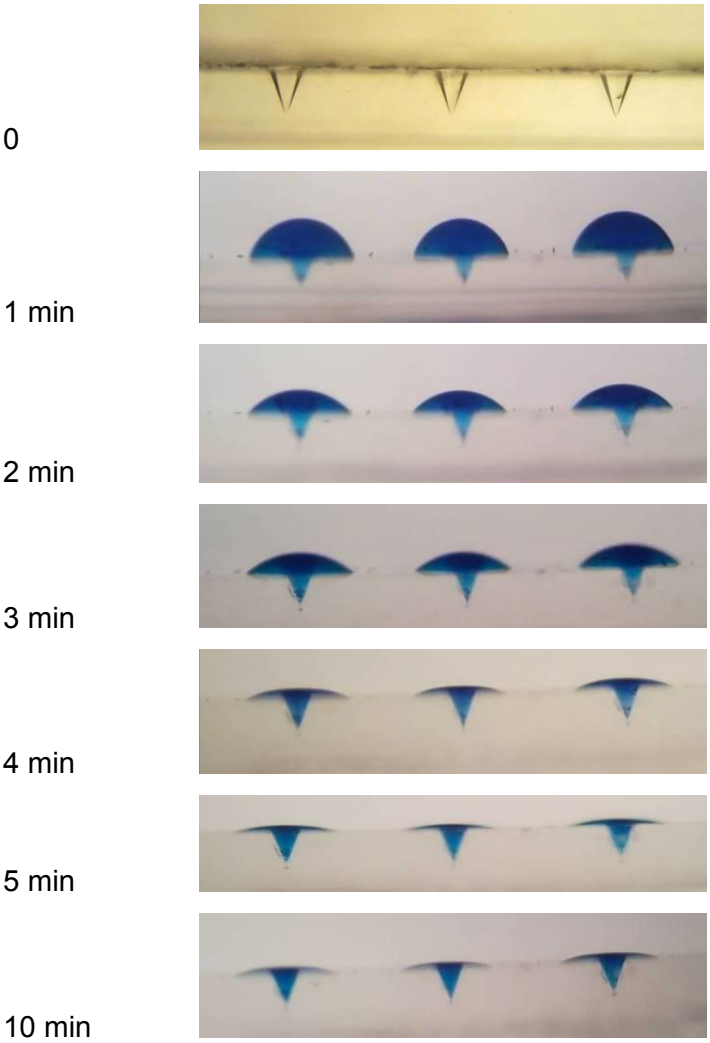

Figure 1d

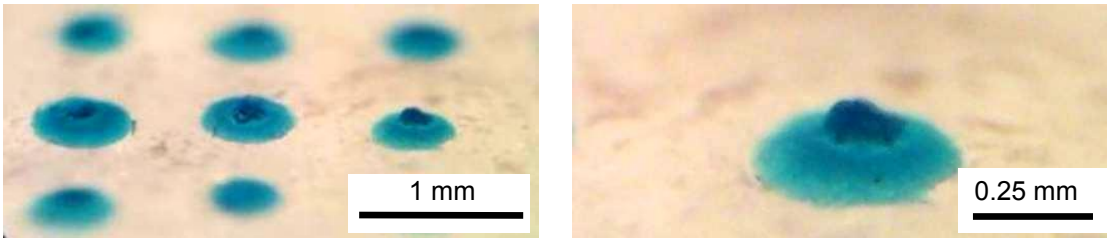

Figure 2.

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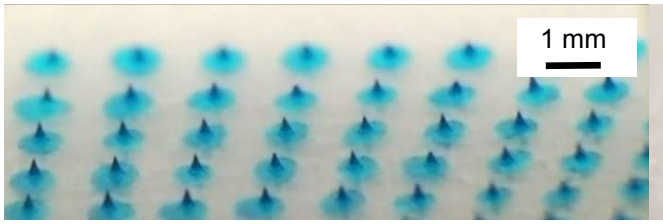

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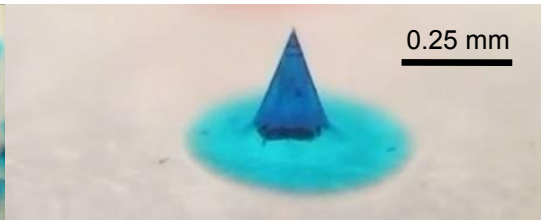

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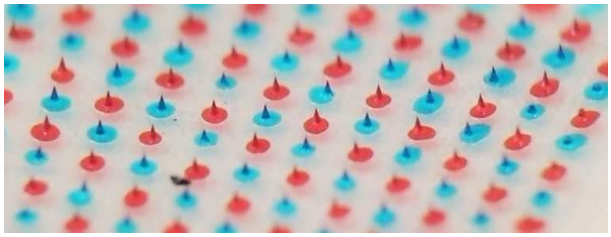

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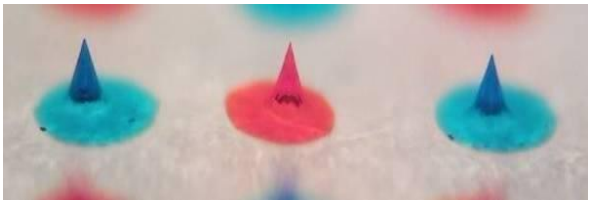


c1

c2

d

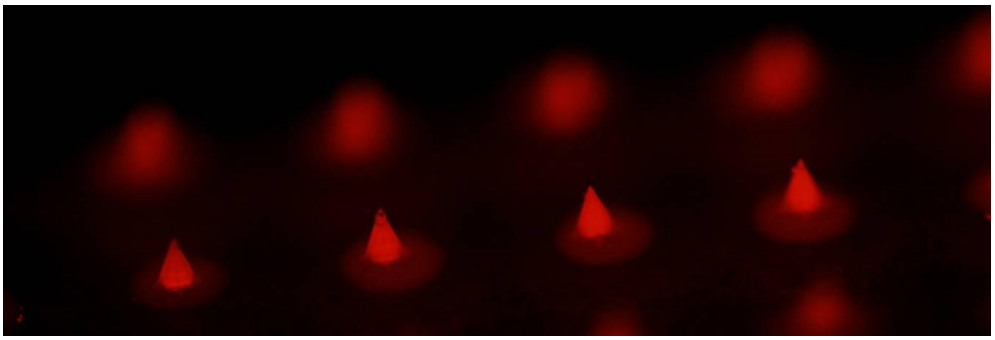


Figure 3.

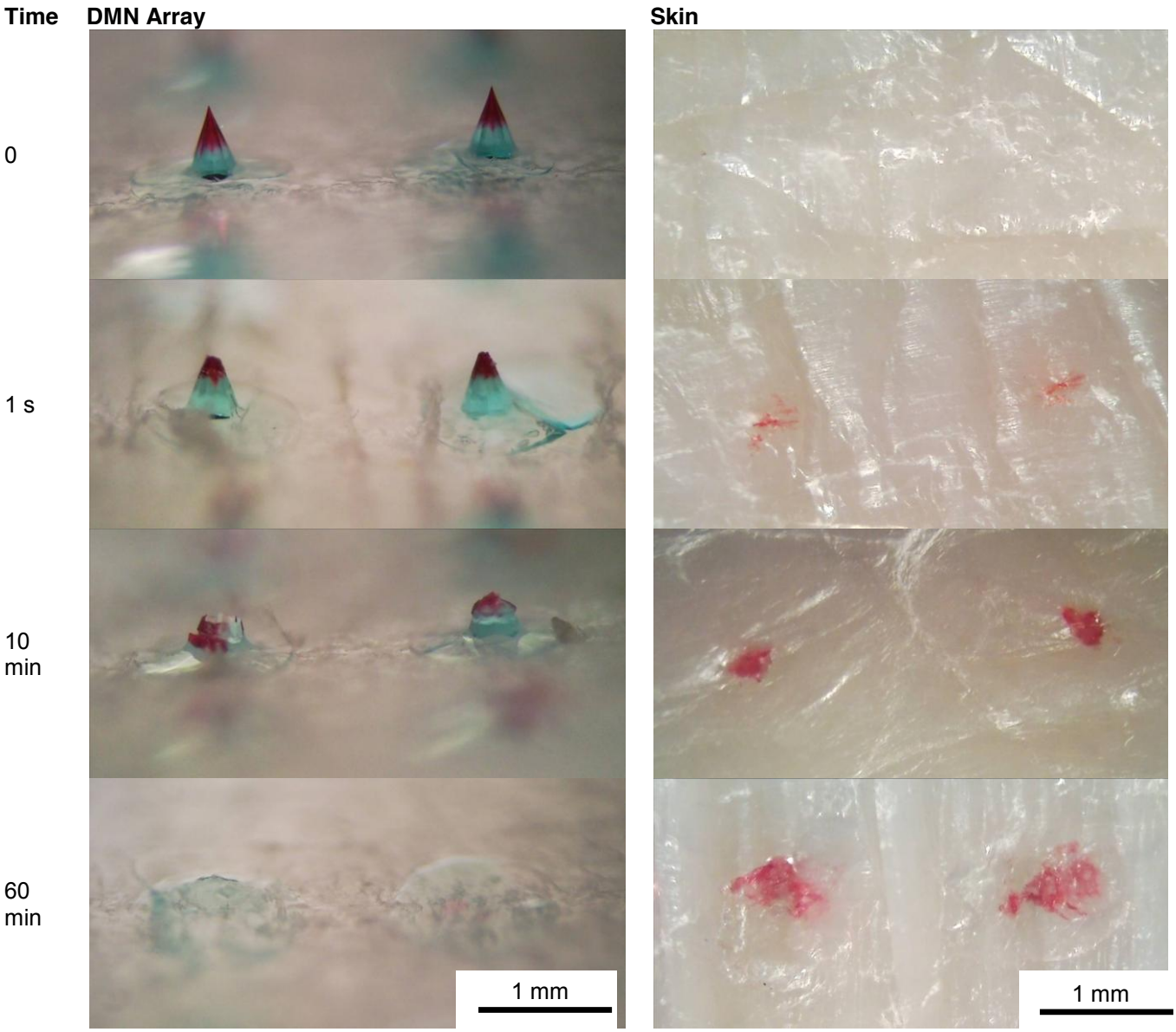


Figure 4.

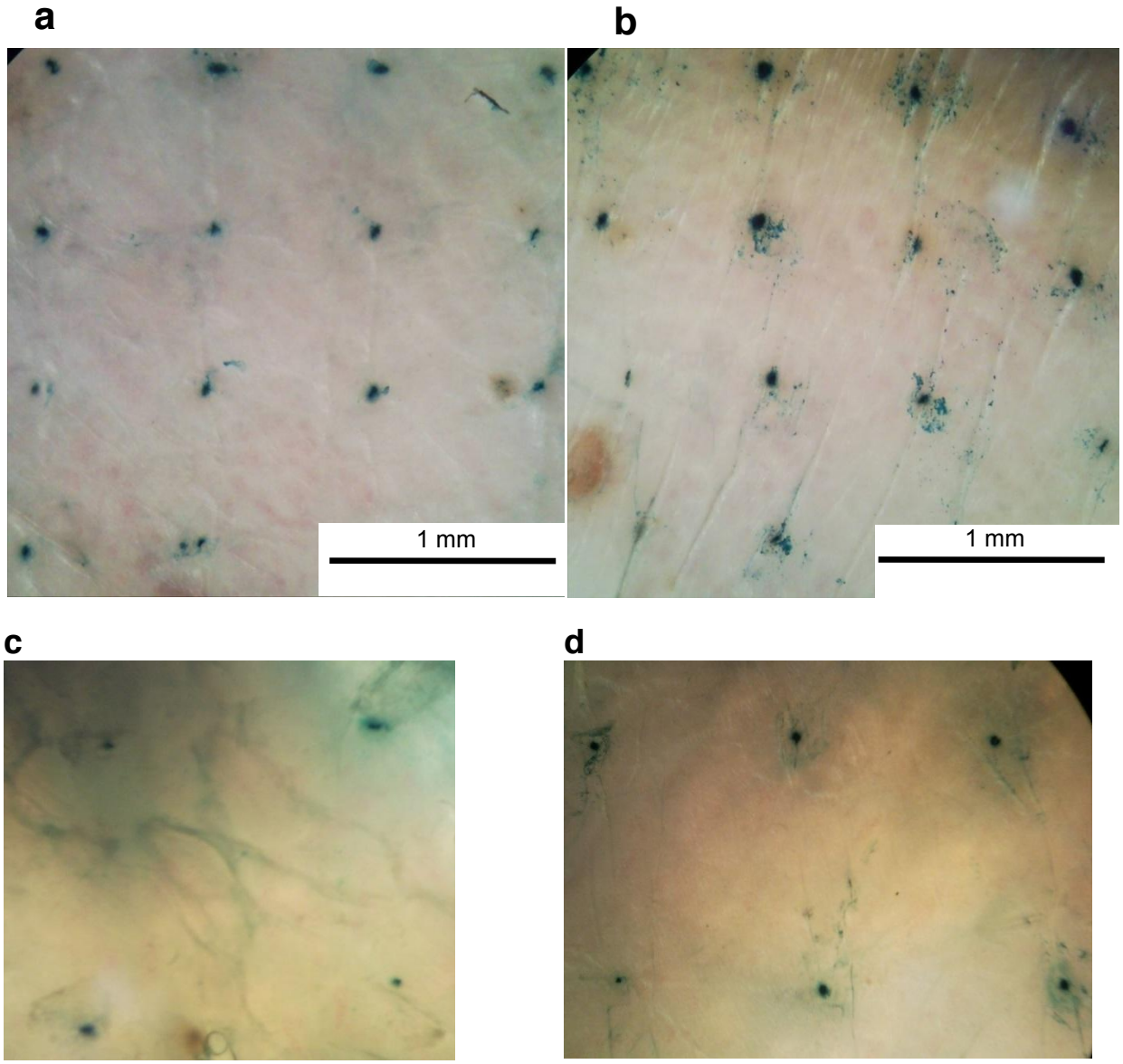


Figure 5.

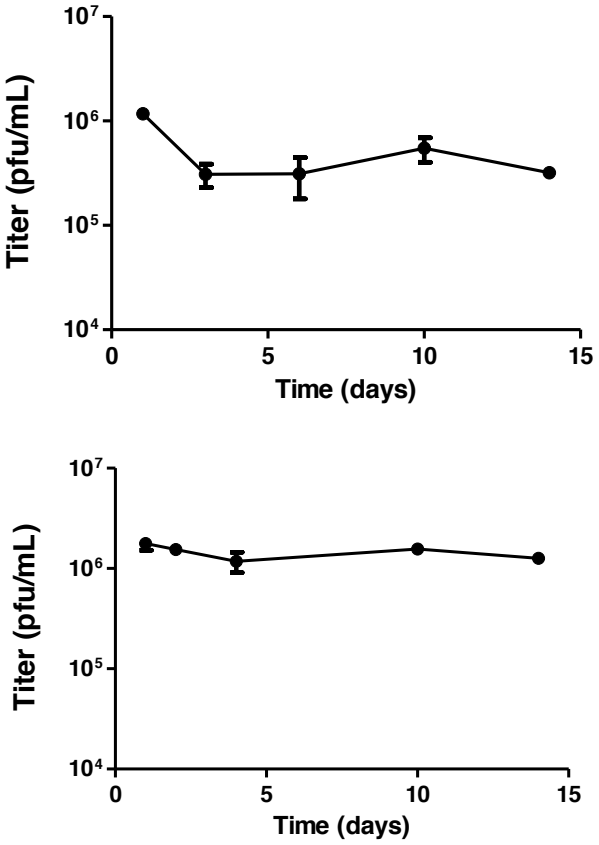
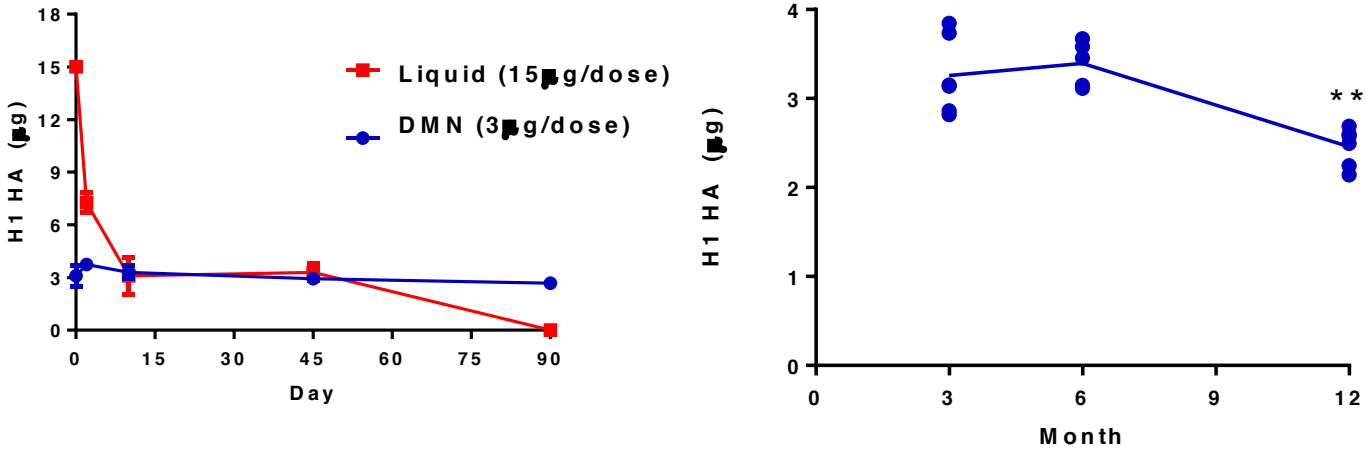

a
10^7
10^6
10^5
10^4
Titer (pfu/mL)
0
5
10
15
Time (days)
b
10^7
10^6
10^5
10^4
Titer (pfu/mL)
0
5
10
15
Time (days)

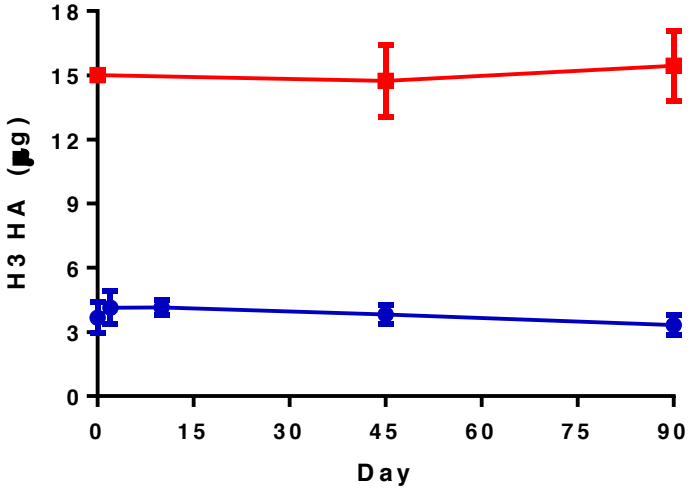
Figure 6

Fig 6

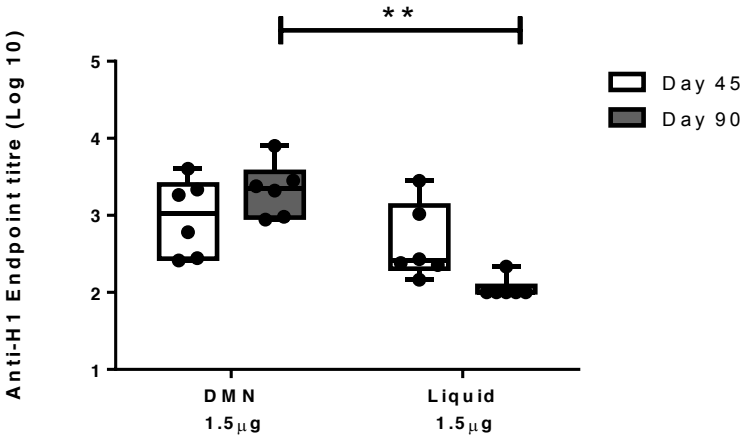
a

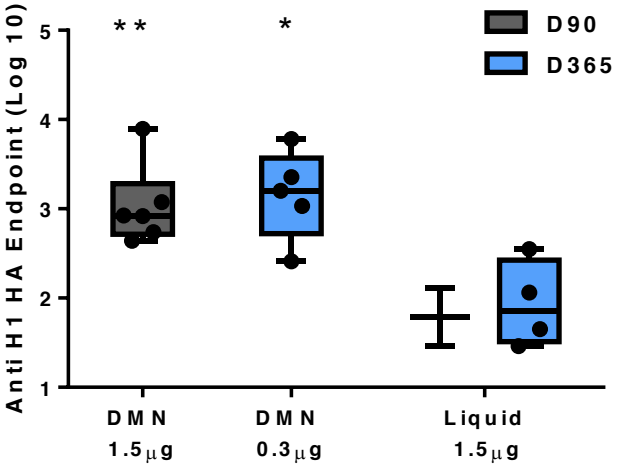


b

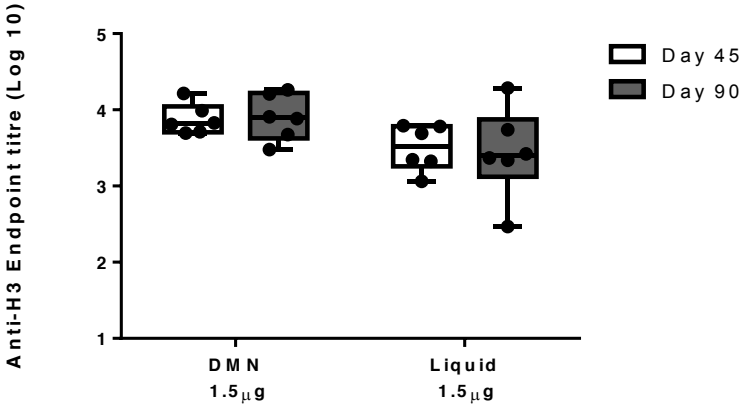


c


Anti-H1 Endpoint titre (Log 10)
**
5
4
3
2
1
DMN
1.5µg
Liquid
1.5µg
Day 45
Day 90


Anti H1 HA Endpoint (Log 10)
**
*
5
4
3
2
1
DMN
1.5µg
DMN
0.3µg
Liquid
1.5µg
D90
D365

d


Anti-H3 Endpoint titre (Log 10)
5
4
3
2
1
DMN
1.5µg
Liquid
1.5µg
Day 45
Day 90

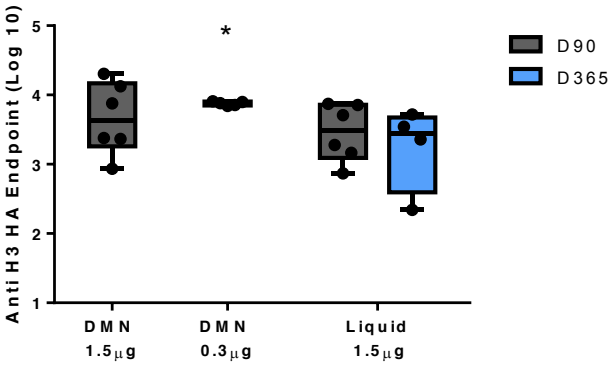
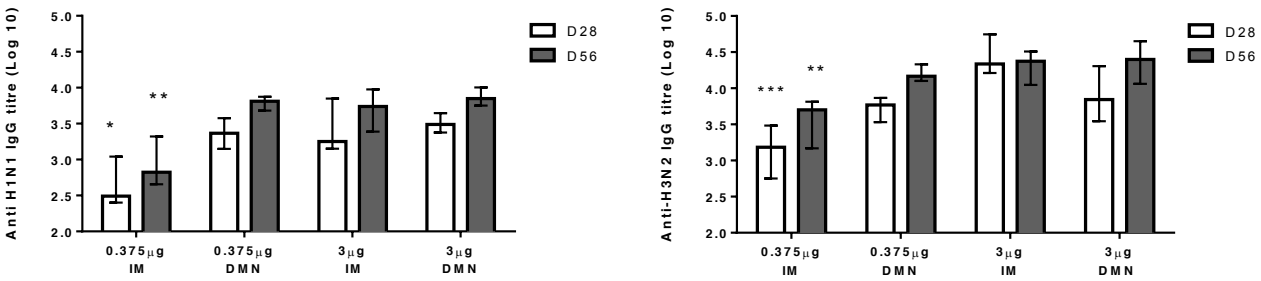
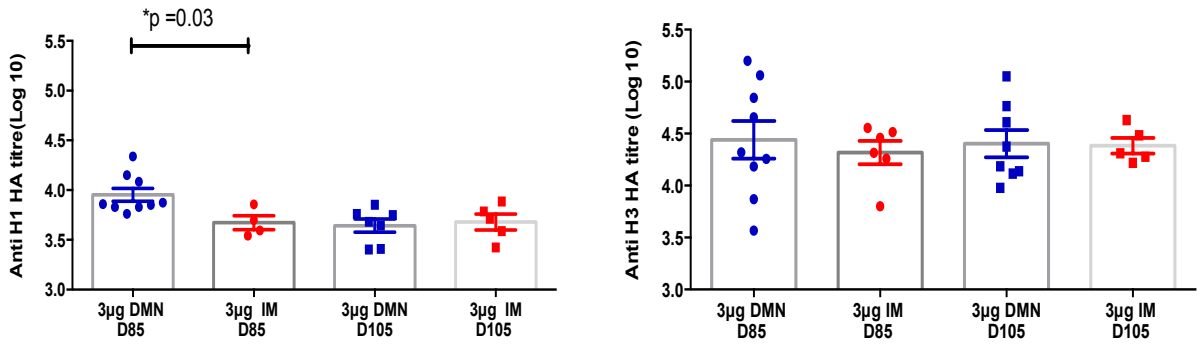

Anti H3 HA Endpoint (Log 10)
*
5
4
3
2
1
DMN
1.5µg
DMN
0.3µg
Liquid
1.5µg
D90
D365

Figure 7

a


Anti H1N1 IgG titre (Log 10)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
*
**
D28
D56
0.375µg IM
0.375µg DMN
3µg IM
3µg DMN
Anti-H3N2 IgG titre (Log 10)
***
**
D28
D56
0.375µg IM
0.375µg DMN
3µg IM
3µg DMN

b


*p =0.03
Anti H1 HA titre(Log 10)
5.5
5.0
4.5
4.0
3.5
3.0
3µg DMN D85
3µg IM D85
3µg DMN D105
3µg IM D105
Anti H3 HA titre (Log 10)
5.5
5.0
4.5
4.0
3.5
3.0
3µg DMN D85
3µg IM D85
3µg DMN D105
3µg IM D105

c

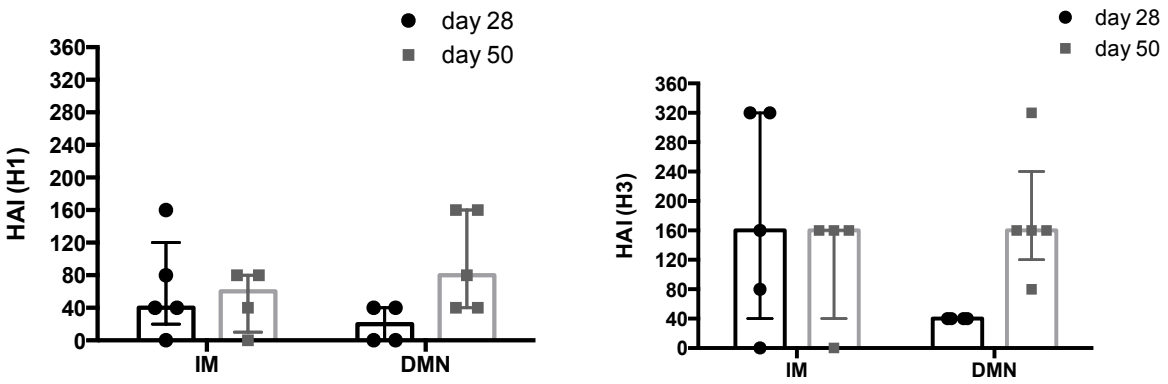
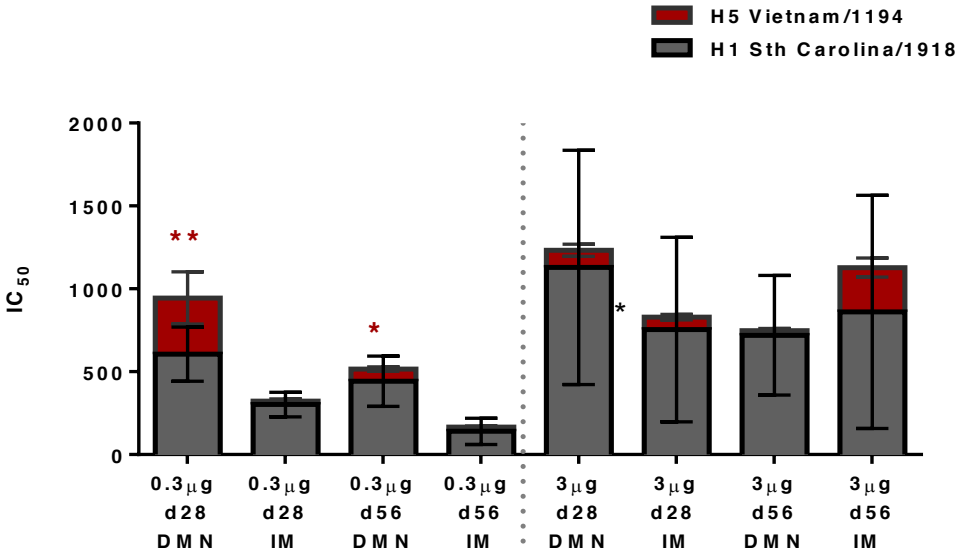
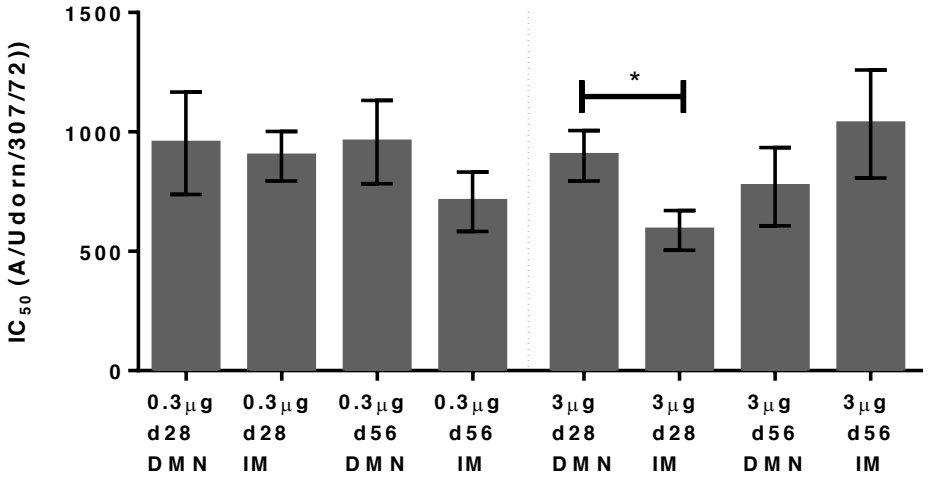

day 28
day 50
HAI (H1)
360
320
280
240
200
160
120
80
40
0
IM
DMN
day 28
day 50
HAI (H3)
360
320
280
240
200
160
120
80
40
0
IM
DMN

Figure 8:

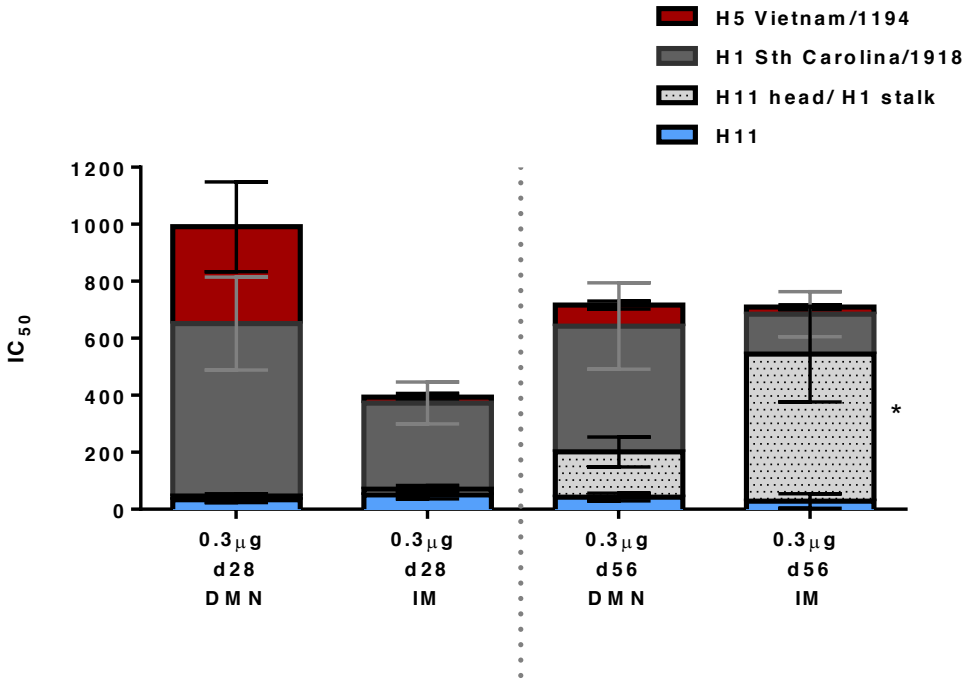
a


H5 Vietnam/1194
H1 Sth Carolina/1918
IC50
2000
1500
1000
500
0
**
*
*
0.3µg d28 DMN
0.3µg d28 IM
0.3µg d56 DMN
0.3µg d56 IM
3µg d28 DMN
3µg d28 IM
3µg d56 DMN
3µg d56 IM

b


IC50 (A/Udorn/307/72))
1500
1000
500
0
*
0.3µg d28 DMN
0.3µg d28 IM
0.3µg d56 DMN
0.3µg d56 IM
3µg d28 DMN
3µg d28 IM
3µg d56 DMN
3µg d56 IM

C


H5 Vietnam/1194
H1 Sth Carolina/1918
H11 head/ H1 stalk
H11
IC50
1200
1000
800
600
400
200
0
*
0.3µg d28 DMN
0.3µg d28 IM
0.3µg d56 DMN
0.3µg d56 IM

Supplementary Video

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Supplementary Figure 1

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